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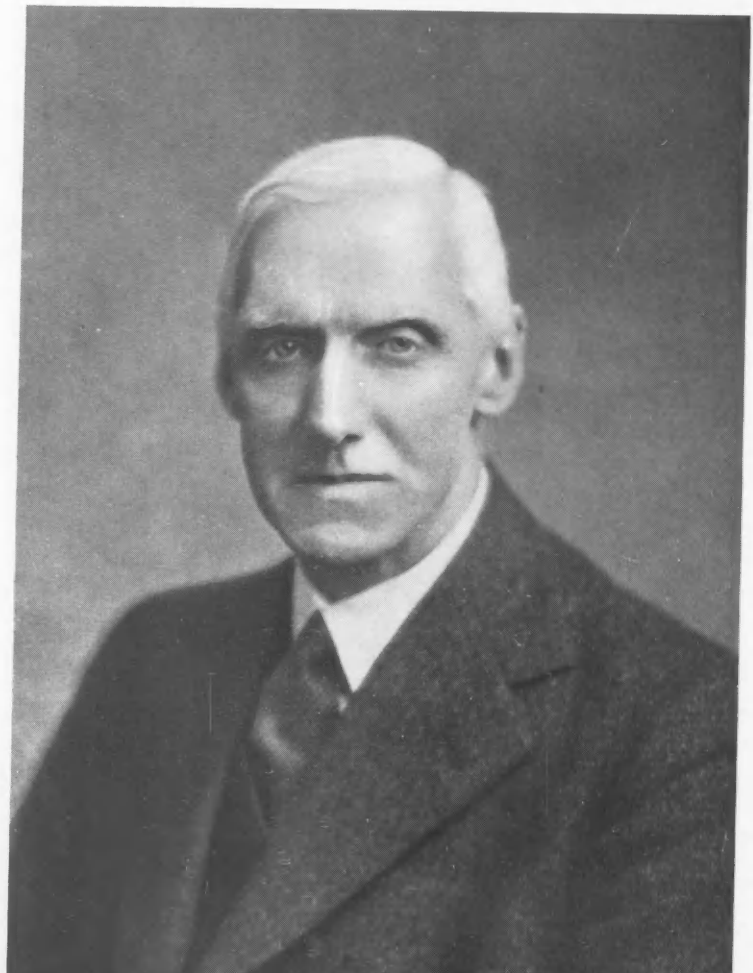

HENRY STANLEY RAPER

Obituary Notice

HENRY STANLEY RAPER
(1882–1951)

Henry Stanley Raper, an eighth child and the youngest of five brothers, was born on 5 March 1882, at Bradford, Yorkshire, where his father managed a large firm of woolcombers. From Belle Vue School, Bradford, he proceeded to Bradford Technical College, and after studying in the Chemistry and Dyeing Department there from the age of 14 to the age of 17 he entered the Yorkshire College, Leeds, in 1899.

Raper graduated with first-class honours in chemistry in the Victoria University in 1903, was awarded a scholarship, and began his researches under the supervision of the late Professor J. B. Cohen. The title of the first publication in which his name appears (with Thompson & Cohen, 1904), 'The Action of Sodium Hypochlorite on Aromatic Sulphonamides', might to-day suggest to us a clash of old-fashioned antiseptic and modern antibacterial; but the matter was then pure organic chemistry. In two papers which were published jointly with Cohen later in this same year we may perhaps see a germ of Raper's subsequent career. These were concerned with the optical activity of the menthyl esters of various halogenated benzoic acids and formed part of Cohen's series on the relation of position isomerism to optical activity. Raper himself has attributed to Cohen at this time a growing belief in the wide scope for chemical investigation provided by the activites of living organisms, a result of his teacher's interest in the mystery of the optical activity of naturally occurring organic compounds. Raper, like Dakin before him, was encouraged by Cohen to turn his attention to physiological chemistry, and in 1904 the award of an 1851 Exhibition scholarship provided the opportunity for his move to the Lister Institute of Preventive Medicine and to J. B. Leathes. Thus he was introduced to what was to prove one of the two main themes of his scientific work, the metabolism of fats, and there began the close association between Leathes and Raper which was most publicly manifested in their joint authorship, in 1925, of the second edition of *The Fats* in the series of 'Monographs on Biochemistry' published by Longmans Green & Co.

Raper's first publication from the Lister Institute described the re-examination of a claim by Hoppe-Seyler (1878) that on heating lactic acid with alkali not only were hydrogen and the lower volatile fatty acids obtained, but also detectable amounts of higher fatty acids. Whilst the formation of acetic acid and smaller amounts of formic, propionic, butyric and *iso*butyric acids under these conditions was confirmed, evidence was provided that the non-volatile products did not in fact include higher fatty acids, and scepticism was expressed as regards Hoppe-Seyler's suggestion that this mode of decomposition of lactic acid might provide a pointer towards the mode of formation of fat from carbohydrate in living organisms. Raper's time at the Lister Institute was divided into two parts by a year (1905–6) spent at Strassburg with Hofmeister. Permanent record of this visit is to be found in a paper 'Zur Kenntnis der Eiweisspeptone' in *Beiträge zur chemischen Physiologie und Pathologie*, but this represented Raper's only excursion into the field of proteolysis, unless one couples with it his introduction of Folley to biochemistry through the medium of the plastein problem in 1926. The β-oxidation theory had been propounded by Knoop in 1904, and complementary to this were the ideas of Magnus Levy and of Leathes that a condensation of acetaldehyde might be the primary reaction of fatty acid synthesis. Available chemical experience seemed, however, to indicate that successive aldol condensations would be more likely to lead to branched-chain than to straight-chain products. Opinion in favour of an aldol-condensation type of chain synthesis was very considerably reinforced by the demonstration by Raper after his return from Strassburg that aldol itself in 5% potassium carbonate solution condensed to form a hydroxyaldehyde which could be oxidized with silver oxide to the hydroxycarboxylic acid; and that the latter on reduction with hydriodic acid gave an acid which was characterized through its crystalline amide as normal octanoic acid, and clearly differentiated from the branched-chain α-ethylhexanoic acid which was prepared synthetically for comparison.

The Lister Institute at this time must have provided a congenial environment for a young biochemist. Martin, Harriette Chick, Dakin, Cathcart, Hedin, Hartley, Harden and Young were at work there, as well as Leathes and several distinguished microbiologists. The year in Germany had increased Raper's acquaintance with biochemistry and biochemists. Yet one may hazard a guess that Raper had soon become convinced that the gulf between organic chemistry and bio-

chemistry was a wide one which could be properly bridged only by the supplementation of chemical by medical studies. Whilst in London he had studied anatomy and histology at King's College; in 1907 he returned to Leeds and in 1910 graduated there in medicine. This was followed by his appointment as Lecturer in Pathological Chemistry in the University of Toronto, and the resumption of research in the field of fat metabolism. Leathes had carried out investigations which led him to conclude that after animals are fed fat the liver takes up fat from the blood and changes its fatty acids so as to increase their iodine value. The experimental data had not shown whether this resulted from a selective uptake by the liver of the more unsaturated acids or from an active desaturation of fatty acids in the liver, and Raper spent much time in an endeavour to devise experiments capable of distinguishing between these alternatives. In this difficult task his characteristic patience must have stood him in good stead, but his equally characteristic honesty compelled him to admit, when describing this work in the *Journal of Biological Chemistry*, that the results were inconclusive.

Shortly after Raper's return to Leeds as Lecturer in Physiological Chemistry in 1913 the first of a group of papers on the mode of oxidation of certain branched-chain fatty acids appeared. The research was interrupted by the first World War which saw Raper first directing the physiology department in Leeds during Professor de Burgh Birch's absence, and later translated to the Anti-Gas Section of the Chemical Warfare Department where he became commanding officer with the rank of lieutenant-colonel. The work on fatty acids was resumed when Raper, now professor of physiology and biochemistry, returned to Leeds in 1919, and in the remaining papers of this group we find for the first time the name of a junior collaborator (in the first instance, H. D. Kay) appearing. These papers dealt with the nature of the products arising from the oxidation *in vitro* by hydrogen peroxide of α-methylbutyric, α-ethylbutyric and α-methylpropionic (*iso*butyric) acids, with the metabolism of hydratropic, tropic, atrolactic and a tropic acids in the dog, andwith the fate in the body of α-methylcinnamic, β-phenyl*iso*butyric and γ-phenyl*iso*valeric acids. The interest in oxidation by hydrogen peroxide derived from Dakin's discovery that this method of oxidation appeared to be analogous to the *in vivo* oxidation of fatty acids as viewed by Knoop, in so far as the main primary product was the β-keto acid. The products obtained from the α-methyl and α-ethyl acids by hydrogen peroxide oxidation, as well as the urinary products previously shown by Blum to arise from these acids in the diabetic animal, were found to be concordant with the view that β-oxidation took place in each case, if the assumption were made that the methyl carbon in the β-position is much more susceptible to oxidation than a methylene carbon in the β-position when they are present together, and is preferentially oxidized to an aldehyde group. The resultant derivative of the semi-aldehyde of malonic acid, it was suggested, would then lose carbon dioxide from its carboxyl group and leave a straight-chain aldehyde with one fewer carbon atom than the parent compound. The *in vivo* experiments with the phenyl-substituted, branched-chain acids gave results in harmony with these views and also provided data which suggested that the αβ-unsaturated acid was not an obligatory intermediate in the β-oxidation of saturated fatty acids.

In 1923 Raper was invited to the Brackenbury Chair of Physiology in the University of Manchester. To Professor Stopford (now Sir John Stopford and the Vice-Chancellor of Manchester) had been delegated the task of visiting Leeds and tendering this invitation, and he has recently recalled his feeling while travelling to Leeds that he might well have great difficulty in the persuasion of one known to have a great love for his native county, and a keen cricketer at that. The success which attended this first official approach between these two, and the mutual liking and respect engendered, were pregnant with good fortune for the University of Manchester. In Manchester, Raper's continued interest in the fats resulted in the appearance in the next few years of a series of papers in collaboration with his students Clutterbuck, Bate Smith, Pearson, Wayne and Dulière. The oxidation of the ammonium salts of fatty acids by hydrogen peroxide, which according to Dakin was essentially a β-oxidation, was now shown to give rise also to significant amounts of γ- and δ-keto acids, in the further oxidation of which some succinic acid appeared. The latter observations suggested a possible mechanism of sugar formation from fatty acid, since succinic acid had already been recognized as glucogenic in the diabetic organism. An attempt was accordingly made to demonstrate the splitting off *in vivo* of a four-carbon fragment of succinic acid type. β-Phenylhexanoic acid was given subcutaneously to dogs in the hope of subsequently isolating phenylsuccinic acid from the animals' urine, the latter acid having been first tested and found resistant to oxidation in the dog. Although only about half of the administered β-phenylhexanoic acid appeared unchanged in the urine, no trace of phenylsuccinic acid could be found. Nor could any formation of succinate be detected when δ-ketohexanoic acid was added to the blood in liver perfusion experiments. Quantitative studies were then made, more accurately than had hitherto

been attempted, of the excretion of benzoic and phenylacetic acids after subcutaneous administration of ω-phenyl fatty acids to the dog. Recoveries of administered benzoic and phenylacetic acids were about 80%. The short-chain ω-phenyl derivatives of acids up to caproic acid also gave urinary benzoic or phenylacetic acid in accordance with Knoop's hypothesis in about 80% of the theoretical yield, suggesting that only β-oxidation was operative; with ω-phenyl-nonoic and -decoic acids, however, the yields were only about 65% and this finding was taken to leave open the possibility that the longer-chain acids might in some degree be susceptible to some other mode of oxidation.

Raper was quick to appreciate the vast possibilities inherent in the application of isotopic tracer techniques in the difficult field of fat metabolism. The new methods became available at a time when his administrative duties had already so multiplied as to leave him little time for research, or even for the supervision of research, but two papers with Cavanagh described the distribution of deuterium in phospholipin and glyceride fractions of various organs of the rat after the animals had been fed with linseed oil partially saturated with deuterium. Incorporation of the fed fatty acids into the liver phospholipins was shown to take place with a rapidity sufficient to support the view that the liver phospholipins participitate in an especially active way in the metablism of fat.

Raper's first biochemical research dealt with fatty acids, and his last scientific work was in the preparation of an introductory address for a projected Biochemical Society Symposium on 'Lipid Metabolism', when he was to occupy the Chair. He was looking forward with the keenest of anticipation to this symposium; but his admirable introduction was read on the occasion, some four weeks after his death, by another chairman called on to take his place.

Biochemists will, however, remember Raper chiefly for the elegant and illuminating series of papers on tyrosinase and the reactions involved in the early stages of melanin formation. This series began from Leeds in 1923, at a time when it had been suggested that during the action of tyrosinase upon tyrosine deamination of the amino-acid took place, and even that tyrosinase could bring about the deamination of other and non-aromatic amino-acids. In the earlier papers, with his students Wormall, Happold and H. B. Speakman, Raper recognized the importance of adequate pH control in experiments on the tyrosinase-tyrosine reaction; showed the subdivision of the overall reaction into (*a*) the oxidative formation from tyrosine of a red substance, (*b*) the spontaneous change of this to a colourless product, and (*c*) the oxidative conversion of the latter to melanin; and provided convincing evidence that liberation of ammonia did not occur. The oxidative deamination of amino-acids such as glycine and alanine was shown to depend on the presence along with the tyrosinase of traces of *p*-cresol or other phenol. Since *o*-benzoquinone was found to deaminate these amino-acids without enzymic aid, the oxidative deamination of them noted by other workers was now revealed as secondary to a tyrosinase-catalysed *o*-quinone formation. In 1926 the isolation of 3:4-dihydroxyphenylalanine (DOPA) from a solution in which mealworm tyrosinase had acted upon tyrosine was announced; and DOPA was shown to undergo further oxidation to melanin more rapidly than, but apparently through the same stages as, tyrosine. The possibility of ring formation by intramolecular change of DOPA-quinone was envisaged, and the nature of the condensation was made clear in 1927 when 5:6-dimethoxyindole and 5:6-dimethoxyindole-2-carboxylic acid were isolated and identified after methylation of the products present in a reaction mixture in which the 'red substance', after removal of the enzyme, had passed to the next, colourless stage. The same products having been obtained with DOPA instead of tyrosine as the starting material, the sequence tyrosine→DOPA→DOPA-quinone→5:6-dihydroxydihydroindole-2-carboxylic acid→*o*-quinone of the latter→5:6-dihydroxyindole-2-carboxylic acid→5:6-dihydroxyindole, was thus established.

In 1927, too, the introduction of aniline into reaction mixtures with simple mono- and dihydroxyphenols as substrates enabled Raper and Pugh to isolate a number of aniloquinones and provide valuable evidence for the view that the essential effects of tyrosinase were the conversion of monophenols into *o*-diphenols, and the oxidation of the latter to the corresponding *o*-quinones. Raper's authority in this field was recognized in the invitation which led to the appearance in 1928 of his valuable article on 'The Aerobic Oxidases' in *Physiological Reviews*. His election to the Fellowship of the Royal Society in 1929 marked the culmination of this period of his most decisive experimentation. It may well be that Raper's interest in the tyrosinase-tyrosine reaction had been stimulated by an idea that analogous reactions might be concerned in the biosynthesis of adrenaline. In further work with Dulière and Heard in which this possibility was explored evidence was obtained of the appearance of small amounts of pressor substance when tyrosinase acted upon *N*-methyl DOPA. The activity of the pressor substance was greatly increased on reduction, the suggestion was made that it was adrenalone, and reaction mechanisms to account for the appearance of adrenalone were postulated.

Though himself gaining distinction in the biochemical world, Raper looked askance at the modern trends which have so widely substituted biochemistry for chemical physiology. He felt that the future of scientific medical practice was endangered by the threat of divorce between biochemistry and physiology, and the strength of his conviction may perhaps partly explain Manchester's reluctance to follow the lead given by other universities towards the establishment of an independent biochemical school. His chief delight was in the laboratory, where he preferred to work himself rather than to direct others. Regretfully, as the years passed, he found the time available for the laboratory severely curtailed, and after the fruitful collaboration with Harper which in 1943 resulted in the identification of a new hormone, pancreozymin, he found himself fully occupied with teaching and administrative duties. Complete honesty and a genial sympathy with his fellow men, abundance of tolerance and tact, these were in him outstanding qualities. They were coupled with a remarkable tenacity of purpose when his considered judgement had been reached. With such qualities it was inevitable that his advice should be widely sought and that his responsibilities should be enlarged. In 1946, reluctantly, he allowed himself to be persuaded to vacate the chair of physiology and to devote himself entirely to the Deanship of the Medical School, and to the host of ancillary duties devolving upon the holder of that office. Manchester will long remain in his debt for his unselfish devotion to the cause of medical education in these last years. The condition of Raper's heart had long been a cause of anxiety to his colleagues and friends, yet he carried on in full harness and with undiminished effectiveness for nearly five years after he had reached the usual retiring age. His impending retirement at Christmas 1951 would have been well-earned, and he was looking forward with quiet pleasure to peaceful days in his garden or at his easel in the countryside. It seemed a cruel fate that, by his unexpected death on 12 December, denied these to him.

L. P. KENDAL

The photograph of the late Professor H. S. Raper is reproduced by kind permission of the Editor, *Medical School Gazette*, the University of Manchester.

Some Aspects of Phosphorus Metabolism in Bone Marrow

2. CHANGES IN THE CONTENT OF PHOSPHORUS COMPOUNDS AND REDUCING SUBSTANCES IN BONE MARROW AND SPLEEN, CAUSED BY IONIZING RADIATION AND OTHER FACTORS WHICH DEPRESS THE FUNCTION OF BLOOD-FORMING TISSUE

By CECILIA LUTWAK-MANN
Biochemical Laboratory, University of Cambridge

(*Received* 12 *February* 1952)

The experiments reported below are a continuation of studies described in an earlier paper (Lutwak-Mann, 1951); the investigation has been extended to provide further information about the behaviour of nucleic acid phosphorus, lipid phosphorus, ascorbic acid and glutathione in rat bone marrow and spleen, under various experimental conditions including dietary changes, total body exposure of normal and splenectomized animals to different single doses of X-rays, thermal skin burns and the action of certain myelotoxic agents and mitotic inhibitors.

MATERIAL AND METHODS

Material. Unless otherwise stated, male rats weighing 180–250 g. were used; the bone marrow collection from the long bones was carried out as previously described (Lutwak-Mann, 1951). Based on the experience gained in earlier studies, an effort was made to use homogeneous groups of animals for any given experimental series, so as to minimize the differences in their response to pharmacodynamic agents.

Methods. The determinations of acid-insoluble P and the abbreviations for the P fractions were the same as before (Lutwak-Mann, 1951). Ascorbic acid was determined by the method of Roe & Kuether (1943), glutathione according to Hopkins & Morgan (1936) and ergothioneine by the method of Hunter (1951).

The effects of dietary changes on the P composition of bone marrow were studied in rats maintained from the age of 6 weeks on the following carbohydrate-free, high-fat diet: arachis oil 75 parts, light white casein 20, salts 5, together with 12 parts of Marmite, either untreated or autoclaved, and the usual daily supplement of cod-liver oil; the animals which received autoclaved Marmite were given parenterally 50 μg. thiamine daily. When transferred to this diet, growth continued for 1 month and thereafter ceased, irrespective of the addition of thiamine; the condition of the coat in these rats was very poor, but the animals ate well and, except for the arrest of growth, showed no other symptoms. Some of

the animals were used at the age of 3·5 months, when their weight did not exceed 100 g., while others were transferred to a full stock diet for a further period of 2 or 4 weeks, when their weights increased by 50 and 85 g., respectively; simultaneously, there was a spectacular improvement in the condition of their coats. Some experiments were also carried out with male rats castrated at the age of 4 weeks and maintained from the third month for a 4-week period either on a full synthetic or on a vitamin B-deficient diet, made up as before (Lutwak-Mann, 1951).

Irradiation experiments were carried out at the Department of Radiotherapeutics, by kind permission of Prof. J. S. Mitchell. Total exposure to single doses of 300, 500, 600 and 800 r. was studied, the physical constants being the same as described earlier. Different batches of rats varied in their susceptibility, particularly to doses above 500 r., not only in the clinical sense, but also in the rate of recovery of the bone marrow P components. Thus for instance, with doses up to 600 r. no haemorrhagic symptoms or deaths were encountered during the 10–14-day routine experimental period, with young adult males of the laboratory stock. For some of the 600 r. irradiation experiments, however, 6–8 month-old female rats were used; several of these developed haemorrhages on the 5th–8th day after irradiation and 30% died within the first 2 weeks. With laboratory stock rats and a dose of 600 r., at least 50% recovery in the bone-marrow nucleic acid P content took place by the 7th day after exposure; but in rats from other sources, sometimes a lower and sometimes a more advanced degree of recovery was found at that time. A dose of 800 r. was lethal to over 75% of rats of all strains, but few succumbed before the 4th day after exposure.

Splenectomies and mock splenectomies were carried out by Dr Pamela Holton at the Department of Physiology, Cambridge; the blood of the operated animals was examined for *Bartonella muris* by Dr P. Tate, Molteno Institute, Cambridge. The incidence of bartonellosis after the removal of the spleen varied greatly; in one series of twenty-seven albino rats none developed the infection throughout a period of several weeks; in the rats of the laboratory stock about 30% succumbed at various times, mostly a few days after operation but sometimes much later. It was possible to prevent the occurrence of early bartonellosis by daily injections of neoarsphenamine; however, in several rats treated in this manner a late macrocytic anaemia developed, although the blood smears were negative for *Bartonella* inclusion bodies. It may be added here that on two occasions acute bartonellosis developed in non-splenectomized rats a few days after exposure to 300 r. X-rays.

Thermal skin burns were produced in the following manner. An area of skin on the back of the animals 2 in. long and 1 in. wide was freed of hair; 2 days later the experiment was carried out under ether-anaesthesia, by firmly pressing to the skin for 30 sec., calico bags of suitable size, filled with 20 g. of dry sand, preheated so as to maintain a temperature of 75°. Superficial and fairly uniform skin burns were produced in this way. Altogether 35 male rats weighing 200–250 g. were used for these experiments. After the heat treatment the animals were placed in individual cages to prevent contamination of the skin. A dry scab usually formed by the 4th–6th day; hair began to regrow from the end of the second week onwards. There was no evidence of discomfort or restriction of movement and the rats ate well and did not lose weight. The routine experimental period did not exceed 2 weeks, but one group of six rats survived a month without a single casualty.

Aminopterin (4-aminofolic acid) and amethopterin (4-amino-N^{10}-methylfolic acid) were made available through the courtesy of Lederle Laboratories Division, American Cyanamid Co., New York, and were given by intraperitoneal injection in single or multiple doses. Colchicine, amidopyrine, and mustard sulphoxide together with dimethyldithiocarbamate, were injected subcutaneously. Muscle adenylic acid, yeast adenylic acid and inosinic acid were injected intraperitoneally as Na salts; only the first two were active pharmacologically in producing within about 1 min. after administration a 'shock'-like condition in the rats, which, however, wore off after 30 min. Yeast adenylic acid appeared more active than muscle adenylic acid. Different batches of rats varied in the extent of their response to these two nucleotides.

RESULTS

Lipid phosphorus content in bone marrow and spleen

The content of lipid P in the bone marrow of rabbits and rats has been determined previously (Lutwak-Mann, 1951); many more determinations have now been carried out both in rat bone marrow and in spleen. Relatively little variation was found, the bone marrow containing 50–60 mg./100 g. (as against 10 mg./100 g. in rat blood), while the spleen contained 60–70 mg./100 g. Bone marrow was found to contain enzymes which cause the breakdown of lipid P; when an aqueous suspension of bone marrow was incubated at 37° for 1 hr., the content of lipid P fell slightly and after a further 3–4 hr. (in the presence of toluene) less than one-third of the original amount remained. Rat spleen behaved similarly.

Ascorbic acid, glutathione and ergothioneine in bone marrow and spleen

Earlier determinations have established the content of glutathione and ascorbic acid in rabbit bone marrow (Lutwak-Mann, 1949). Numerous analyses were now carried out to determine ascorbic acid in rat bone marrow. In fresh tissue an average content of 24 mg./100 g. was found, estimated as oxidized ascorbic acid (Roe & Kuether, 1943); estimations carried out by means of the 2:6-dichlorophenolindophenol titration gave very similar values. Although there is a delay in the collection of bone marrow, it is unlikely that this could affect significantly the level of ascorbic acid, as it was found that small amounts of ascorbate well mixed into bone marrow and left at room temperature for 15 min. could be quantitatively recovered. The average content in rat spleen was 36 mg./100 g. However, in some batches of rats as much as 51 mg. ascorbic acid/100 g. was found in the spleen.

For the iodometric assay (Hopkins & Morgan, 1936) of glutathione in bone marrow, material from six to eight rats was pooled; fairly satisfactory agreement was found in three such series, the values being 198, 150 and 165 mg./100 g. Rat spleen contained 165–180 mg. glutathione/100 g.

Ergothioneine was determined in 1·4 g. rat bone marrow which was found to contain 2·7 mg./100 g.; rat spleen contained less than 1·0 mg./100 g.

The effect of dietary changes on the phosphorus composition of bone marrow

Previous studies were concerned with the effect upon bone marrow of quantitative restriction of normal food and of food deficient in vitamin B. In the present series of experiments the animals were maintained on a high-fat diet which had an adequate calorie as well as protein content, and which was not deficient in vitamins; it was, however, poorly balanced owing to the excess of one constituent, liquid arachis oil, and it failed to support normal growth, regardless of whether the animals received untreated Marmite, or autoclaved Marmite plus a daily supplement of 50 μg. thiamine. Table 1 gives the P distribution in the bone marrow of rats at the age of 3·5 months, that is, after 8 weeks of the high-fat diet; it is clear that considerable changes have taken place not only in nucleic acid but also in lipid P. However, it was found that the damage was not permanent, because when some of these animals were transferred to a full stock diet for relatively short periods of 2–4 weeks, considerable improvement in the chemical composition of the bone marrow was noted, parallel with the resumption of growth.

Here may be mentioned an experiment with forty male rats castrated at the age of 4 weeks, and kept thereafter on a full stock diet until they reached the age of 3–4 months. It was found that the bone marrow of the castrates did not differ in its P pattern from that of non-castrate male rats. When the castrated animals were placed for 1 month on a vitamin B-deficient diet, the same changes occurred in their bone marrow as had previously been described for non-castrate rats (Lutwak-Mann, 1951). Testosterone propionate, while it had its usual activating effect upon the accessory organs of the vitamin B-deficient castrates, did not influence the bone marrow composition in any significant manner.

The effect of total body exposure to different doses of röntgen radiation on the content of phosphorus, ascorbic acid and glutathione in bone marrow and spleen

Following the exposure to 300, 500, 600 and 800 r., the bone marrow and spleen were analysed (*a*) within a few minutes after irradiation, (*b*) 6 hr. later and (*c*) 2, 7 and 10 days later, for their content of nucleic acid and lipid P, ascorbic acid and, in some experiments, glutathione. The changes observed in the content of nucleic acid and lipid P in the bone marrow of laboratory stock young adult male rats exposed to 300, 600 and 800 r., are seen in Fig. 1. Except with the highest dose employed, it was not possible to register the changes which presumably occurred immediately after irradiation, by means of the chemical methods used in this study. However, after 6 hr. there was already a pronounced breakdown of nucleic acid P, even with the relatively small dose of 300 r., whereas lipid P remained practically unaltered. Two days after exposure to 800 r., the composition of the material removed from the long bones resembled blood, rather than bone marrow; a considerable degree of loss in nucleic acid and lipid P was also caused by 600 r.; 300 r. did not affect lipid P very much, but nucleic acid P was well below half its initial value. At 7 days, there was marked recovery after the smallest dose; the majority of the rats irradiated with 600 r. showed at least a 50 % recovery at that stage and in those which survived 800 r. there was a trend towards recovery. Animals were not examined at 10 days following exposure to 800 r.;

Table 1. *Nucleic acid and lipid phosphorus content in the bone marrow of male rats, maintained on a high-fat diet*

(Groups 1–3 contained four rats each; group 4 contained two rats. Values given are mean and range.)

Group no.	Diet	(mg. P/100 g.) DNA	RNA	Lipid
1	High-fat diet from the age of 6 weeks to 3·5 months	86 (78–93)	50 (48–52)	37 (31–44)
2	Same period of high-fat diet as in group 1, followed by 2 weeks of full stock diet	120 (110–132)	66 (59–77)	44 (42–46)
3	Same period of high-fat diet as in group 1, followed by 4 weeks of full stock diet	155 (148–163)	75 (71–77)	58 (58–59)
4	Full stock diet for 4 months	168 (166, 170)	73 (72, 73)	58 (56, 60)

those which had received 300 r. were almost fully recovered while those exposed to 600 r. also showed considerable improvement over the preceding stage.

large doses of radiation, the loss in nucleic acid P content seldom amounted in the spleen to more than 30 or 40% of the control value. Recovery also appeared more rapidly than in the bone marrow,

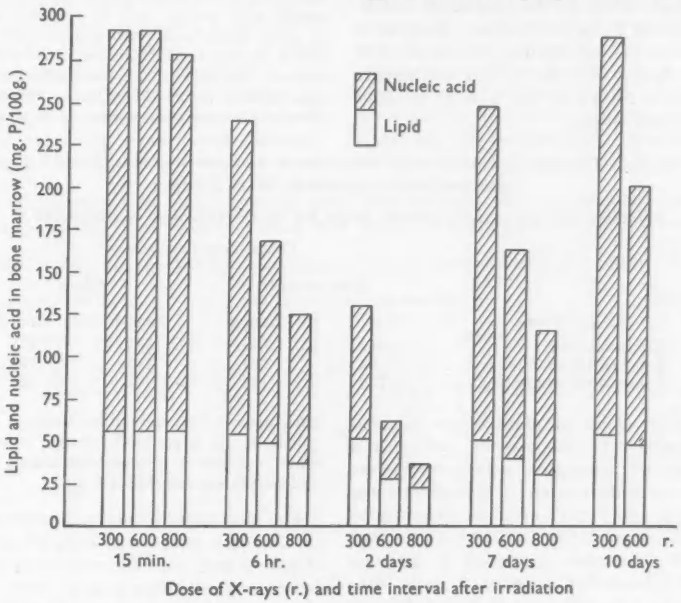

Fig. 1. The behaviour of nucleic acid and lipid P in the bone marrow of rats exposed to 300, 600 and 800 r. X-radiation Blank areas represent lipid, shaded areas nucleic acid P.

Table 2. *Effect of X-rays on the content of nucleic acid and lipid phosphorus, and ascorbic acid in bone marrow and spleen*

(Each value is the average for three rats. Exp. 1 was carried out with male albino rats; Exp. 2 with male hooded rats.)

Exp. no.	Dose of radiation (r.)	Time after exposure to X-rays	Nucleic acid (mg. P/100 g.)		Lipid (mg. P/100 g.)		Ascorbic acid (mg./100 g.)	
			Bone marrow	Spleen	Bone marrow	Spleen	Bone marrow	Spleen
1	0	—	233	152	58	62	26	48
	300	6 hr.	137	137	50	59	18	—
	300	2 days	128	110	48	54	12	38
	300	7 days	159	125	60	60	22	42
	0	—	248	128	55	69	23	36
	800	6 hr.	78	97	39	60	12	31
	800	2 days	12	79	24	52	7	26
	800	7 days	100	112	33	—	15	35

With regard to the behaviour of the spleen in the irradiated animals, the following observations were made; the changes in nucleic acid and lipid P content were less pronounced than those in the bone marrow, with exposure to radiation up to 500 r.; above this amount, there was a distinct fall in the content of nucleic acid P, at 2–4 days after irradiation, but lipid P remained relatively unchanged. Even with

but it was difficult to assess its extent owing to the spectacular changes in weight which rat spleen undergoes in response to radiation, so that while the concentrations might return to the pre-irradiation level, the amount per organ might be considerably less than in untreated animals. In Table 2 results are given for bone marrow and spleen following exposure to 300 and 800 r.

Table 2 also contains data for the amount of ascorbic acid in these two tissues. It can be seen that 50 % or more of the original content of ascorbic acid was lost from the bone marrow at the post-irradiation stage corresponding to the maximum breakdown of nucleic acid P; there was also a decrease in spleen but to a much smaller extent. The recovery in the P content during the later period was always accompanied by a return of the level of ascorbic acid to near-normal values.

Table 3. *Content of glutathione in bone marrow and spleen of rats, 2 and 7 days after total body exposure to 500 r. X-rays*

(Material from six to eight rats was pooled for the determination of glutathione.)

	Glutathione (mg./100 g.)			
	Bone marrow		Spleen	
Group of rats	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Non-irradiated	198	150	165	172
2 days after irradiation	84	110	115	130
7 days after irradiation	155	128	160	148

The iodometric assay of glutathione required large quantities of rat bone marrow, and only a limited number of irradiation experiments were carried out in which the content of glutathione was determined at 2 and 7 days after an exposure to 500 r. The values recorded in Table 3 indicate that the amount of glutathione decreased in the bone marrow of the irradiated animals 2 days after exposure while at 7 days the values were not much below the controls. Results with spleen were less clear; in one experiment the trend was analogous to that in bone marrow, but in the other the change appeared insignificant. It is possible that other animals may react more vigorously in this respect and that more profound changes might be observed in the content of glutathione with larger or repeated doses of radiation.

The effect of muscle adenylic acid, yeast adenylic acid and inosinic acid on the rate of nucleic acid phosphorus recovery in the bone marrow and spleen of irradiated rats

It was stated above that laboratory stock rats exposed to 600 r. radiation consistently showed, at 7 days, at least 50 % recovery in their content of nucleic acid P in the bone marrow, and nearly normal values in the spleen. An attempt was made to influence the rate of recovery in the following manner.

Three series of rats each containing fourteen to eighteen animals were used; four animals in each series were used as controls while the rest were injected daily with 15 μg./g. of muscle adenylic acid, yeast adenylic acid or inosinic acid, for 4 days before irradiation. On the day of irradiation, half of this group were given an injection immediately before exposure, and thereafter for 5 consecutive days, whereas the remainder, including the untreated controls, were irradiated but did not receive any further injections. All animals were examined on the 7th day after irradiation. At autopsy it was found that the rats which had received muscle or yeast adenylic acid both before and after irradiation, but not those treated with inosinic acid, had a bone marrow which in its gross appearance resembled an early post-irradiation period, in that it was fluid and contained blood, while in animals which had either not been injected at all or had only been injected before the exposure, the bone marrow was already of almost normal firm consistency, without blood being present. The only gross change in the spleen was in weight: it was larger (580–650 mg.) in the group treated before and after irradiation with muscle or yeast adenylic acid than in the rest (380–480 mg.).

The quantitative results concerning P composition, as well as ascorbic acid, are given in Table 4. It can be seen that the continuous treatment with muscle or yeast adenylic acid distinctly retarded the recovery of the bone marrow and spleen, as judged by the level of the substances determined in these experiments; inosinic acid had no comparable effect. Administration of either of the two active nucleotides before irradiation only, appeared to have either no effect on the rate of recovery, or only a slight one, in some rats pre-treated with yeast adenylic acid. It must be added here that in separate experiments it was ascertained that eight consecutive injections of muscle or yeast adenylic acid, not followed by irradiation, had no significant effect upon the P content of rat bone marrow.

For the present, the author has no opportunity to extend these experiments sufficiently to assess fully their significance; they are, however, quoted both for their inherent biological interest as well as to underline the usefulness of quantitative chemical assays in the examination of haemopoietic tissue.

Effect of splenectomy on the phosphorus pattern of bone marrow

These experiments have been carried out in collaboration with Dr Pamela Holton, who splenectomized fifty rats. In one-third of the animals the content of nucleic acid and lipid P was determined at various stages between 2 weeks and 2 months after the operation; the remainder of the splen-

ectomized animals were used for irradiation experiments, in which they were exposed to single doses of 300 and 500 r. of radiation, at 1, 2, 3, 6 and 7 weeks after splenectomy.

It was found that the bone marrow of the splenectomized animals showed the same P pattern as that established for normal animals. On several occasions the bone marrow was examined in rats which as the result of splenectomy suffered from acute bartonellosis; in these the ribonucleic acid (RNA) value in the bone marrow was distinctly higher than the highest recorded for normal rats, i.e. 110 mg./100 g. against 85–95 mg./100 g. No changes were observed, however, in the content of deoxyribonucleic acid (DNA) or lipid P in the bone marrow. The only other instance where similarly elevated RNA values were observed was in the bone marrow of rats infected with *Trypanosoma lewisi* (placed at the author's disposal by Dr J. Ryley).

As regards the effect of irradiation on splenectomized rats, here too the changes observed in the bone marrow were indistinguishable from those found in the control unoperated or mock-splenectomized rats, irradiated simultaneously with the splenectomized animals.

The effect of skin burns on rat bone marrow and spleen

To determine whether substances released from burned skin are capable of altering the chemical composition of bone marrow or spleen, experiments were carried out in which adult male rats were examined at various stages after the exposure of a limited area of the skin to 75° for 30 sec. Analyses of bone marrow and spleen failed to produce evidence of any significant changes in these tissues.

Table 4. *Effect of treatment with muscle adenylic acid, yeast adenylic acid and inosinic acid on the bone marrow and spleen of rats irradiated with X-rays* (600 r.) *and examined 7 days after irradiation*

(The amount of nucleotide injected daily was 15 μg./g. The values represent averages; range in brackets.)

Exp. no.	Nucleotide injected	Daily doses: Before irradiation	Daily doses: After irradiation	Rat no.	Bone marrow: DNA (mg. P/100 g.)	Bone marrow: RNA (mg. P/100 g.)	Bone marrow: Ascorbic acid (mg./100 g.)	Spleen: DNA (mg. P/100 g.)	Spleen: RNA (mg. P/100 g.)	Spleen: Ascorbic acid (mg./100 g.)
1	None	0	0	4	102 (90–116)	67 (62–69)	11 (10–12)	64 (55–79)	60 (53–61)	29 (26–34)
	Muscle adenylic acid	4	0	7	89 (61–102)	62 (58–67)	11 (10–13)	66 (57–69)	66 (58–69)	—
		4	5*	7	28 (16–47)	37 (24–40)	6 (4–8)	45 (37–54)	49 (40–56)	19 (17–22)
2	None	0	0	4	116 (88–132)	68 (60–71)	12 (9–15)	68 (60–72)	63 (58–68)	34 (29–37)
	Yeast adenylic acid	4	0	6	88 (59–97)	57 (40–69)	11 (8–12)	55 (52–65)	62 (58–68)	—
		4	5*	7	23 (6–39)	24 (11–49)	6 (4–8)	44 (39–49)	53 (42–56)	22 (18–25)
3	None	0	0	4	111 (89–122)	66 (59–71)	11 (9–12)	66 (57–70)	59 (51–63)	31 (29–36)
	Inosinic acid	4	0	4	115 (92–124)	68 (61–76)	11 (9–13)	—	—	—
		4	5	6	121 (98–129)	70 (60–75)	14 (11–16)	79 (56–82)	63 (49–68)	33 (21–37)

* Bone marrow fluid, with considerable admixture of blood.

The effect of aminopterin, amethopterin, colchicine and amidopyrine on the content of phosphorus and ascorbic acid in bone marrow and spleen

The previous study (Lutwak-Mann, 1951) demonstrated the striking effect upon the nucleic acid P content in the bone marrow of rats suffering from systemic poisoning with mustard gas or mustard sulphoxide combined with dimethyldithiocarbamate. It was of interest to investigate the effect of other myelotoxic agents and to compare their action with that of ionizing radiation.

Table 5 gives the results obtained with parenteral doses of aminopterin ranging from 0·18 to 0·5 μg./g.

Table 5. *Effect of aminopterin, amethopterin, mustard sulphoxide + dimethyldithiocarbamate, and colchicine on the content of nucleic acid* P, *lipid* P *and ascorbic acid in rat bone marrow and spleen*

(Each value represents the average for two to three rats. Male rats used in Exp. 1; female rats in Exp. 2.)

							Bone marrow				Spleen			
Exp. no.	Substance injected	Daily dose (no.)	Daily dose (μg./g.)	Interval after last dose (hr.)	Condition of animals	Gross appearance of bone marrow	DNA (mg. P/ 100 g.)	RNA (mg. P/ 100 g.)	Lipid (mg. P/ 100 g.)	Ascorbic acid (mg./ 100 g.)	DNA (mg. P/ 100 g.)	RNA (mg. P/ 100 g.)	Lipid (mg. P/ 100 g.)	Ascorbic acid (mg./ 100 g.)
1	None	—	—	—	Good	Normal (coral pink, firm)	152	80	60	26	107	65	68	51
	Aminopterin	7	0·18	1	Fair	Very pale, rather firm	82	43	58	20	86	54	60	52
		8	0·25	24	Fair	Thin, blood admixture	68	39	40	18	94	50	—	50
		1	1·70	5	Good	Normal	128	70	57	18	104	58	58	48
		2	1·50	120	Moribund	Very thin, large admixture of blood	35	20	25	7	92	53	—	39
		2	0·50	168	Poor	Colourless, firm	47	55	40	10	89	59	—	45
	Amethopterin	4	1·60	24	Fair	Speckled, fatty	145	74	53	16	101	56	—	49
		6	1·60	24	Fair	As above	94	46	52	13	109	50	58	50
	Mustard sulphoxide + dimethyl dithiocarbamate	1	20 + 100	20	Fair	Very thin, large admixture of blood	55	70	43	12	79	51	—	38
		1	20 + 100	40	Poor	As above	25	26	40	9	66	46	52	40
2	None	—	—	—	Good	Normal	162	88	52	20	132	66	66	32
	Colchicine	1	2·00	8	Poor	Dark red, jelly-like	106	52	42	10	124	55	61	27
		1	2·00	24	Poor	As above	72	32	48	5	111	46	50	26
		1	15·00	24	Moribund*	As above	65	21	34	8	117	54	57	25

* Spleen weight 280 mg. as compared with average weight of 800 mg. in the normal rats.

body weight, at different stages of the treatment. Rats appeared capable of withstanding multiple small doses of aminopterin which, though they did not provoke clinical manifestations, nevertheless led to a considerable depression in the level of nucleic acid P in the bone marrow without, however, affecting the spleen. A single large dose of aminopterin did not produce obvious changes within 5 hr.; but two such doses proved lethal if the animals were left sufficiently long for the general symptoms to develop; again, it was found that the bone marrow was selectively affected. A striking feature, not observed after other treatments, e.g. X-radiation, was the gross appearance of the bone marrow in the later stages of aminopterin treatment, when it was almost colourless, although of almost normal firm consistency. Lipid P was little changed after small doses, but fell significantly after the administration of larger amounts. Loss in the content of ascorbic acid accompanied the decrease in P content of the bone marrow.

Amethopterin was much less toxic to rats than aminopterin; its effects were also confined to the bone marrow (Table 5).

A single dose of colchicine (2 μg./g. body weight) produced a considerable depression in nucleic acid P of the bone marrow 8 hr. after injection; a further decrease was noted at 24 hr. Colchicine appeared to affect the RNA fraction even more severely than DNA, especially in the experiment with a lethal dose of 15 μg./g. The effect on ascorbic acid was pronounced, but on lipid P it was relatively slight. Rat spleen reacted to colchicine treatment by a fall in weight sometimes to one-third of the control value, but the chemical changes were not striking.

Table 5 records also some experiments with mustard sulphoxide plus dimethyldithiocarbamate; the effect of these two substances on the bone marrow P has been demonstrated earlier; it was found now that, as with X-rays, it was not limited to the bone marrow but extended also to the spleen. There was also a decrease in the content of ascorbic acid, more pronounced in the bone marrow than in spleen.

On the other hand, amidopyrine, which is known to inhibit rat bone marrow glycolysis *in vitro* (Lutwak-Mann, 1949), failed to produce changes in the bone marrow or spleen, even after prolonged treatment with amounts up to 0·1 mg./g. daily.

DISCUSSION

The findings made in the course of this study have demonstrated a difference in the susceptibility of important tissue constituents to the impact of certain physical and chemical agents applied to the animal as a whole. Thus, with suitably graded doses of röntgen radiation, it was possible to produce a major breakdown of nucleic acid in the bone marrow and spleen, without affecting the content of lipid P to any marked extent. In general, the reactivity of the nucleic acid P towards a variety of agents has proved to be much greater than that of the lipid P; the changes in nucleic acid P were accompanied in all instances by a decline in the content of ascorbic acid and presumably also of glutathione, though with regard to the latter, a further strengthening of the experimental evidence is desirable.

In this connexion it may be recalled that earlier attempts to correlate the glutathione and ascorbic acid content of organs such as liver with the extent of the X-ray injury were inconclusive (Lutwak-Mann, 1951), although marked changes in the content of these reducing substances do occur in the liver in response to pharmacologically active agents, such as salicylates and similar aromatic substances (Lutwak-Mann, 1942). Nevertheless, the depletion in ascorbic acid of the bone marrow and to a lesser extent of the spleen may be taken to be established as a result not only of irradiation, but also of the action of chemically unrelated substances, such as mustard gas, aminopterin and colchicine.

It is interesting to record in blood-forming tissue, in addition to ascorbic acid and glutathione, the presence of yet another reducing substance, ergothioneine, which has also been found in red blood corpuscles (Hunter, 1951) and in the reproductive tract (Leone & Mann, 1951).

The comparative study of bone marrow and spleen has indicated a qualitative similarity of their behaviour in most, if not in all, experimental conditions: whereas exposure to X-rays or to mustard sulphoxide induced analogous, though quantitatively different, chemical changes, the folic acid antagonists and, to a smaller extent, colchicine appeared to act selectively on the bone marrow. However, before attempting to evaluate the effect of any given agent on spleen, in relation to the whole animal, account must be taken of the conspicuous shrinkage of this organ at certain stages of the experimental period.

By selecting homogeneous groups of experimental animals, it was possible to establish satisfactorily the extent of nucleic acid P recovery in bone marrow and spleen, at an arbitrarily chosen stage, 7 days, following exposure to 600 r. X-rays. An attempt was then made to influence this process by subjecting the animals to treatment with muscle adenylic acid or yeast adenylic acid, before and after irradiation. Both the gross appearance and the chemical analyses, particularly of bone marrow, but also of spleen, at 7 days after irradiation, pointed to an interference with the recovery of these tissues in the nucleotide-treated animals. While much further work is necessary to assess the significance of these results, it is instructive to find that only the nucleotides with an intact adenine-

amino group appeared active, inosinic acid being devoid of potency in this respect. It is known from earlier researches, notably those of Bennet & Drury (1931), that muscle adenylic acid and yeast adenylic acid, administered intraperitoneally, produce leucopenia followed by leucocytosis. It therefore seems not unlikely that prolonged stimulation of the blood-forming tissue by suitably timed treatment with these nucleotides may, at any rate in a certain proportion of animals, modify the response to ionizing radiation and possibly to other myelotoxic agents as well.

A further instance of the deleterious effect which a poorly balanced diet may exert upon the bone marrow was provided by experiments with the high-fat diet which, although not deficient in vitamins or protein, failed to promote growth and led to a remarkable diminution in the content not only of nucleic acid but also of lipid P. On the other hand, gonadectomy, splenectomy or mild-degree skin burns, produced no major change in the P pattern of rat bone marrow. As with all such work, the possibility remains that other animal species would react differently under such circumstances, especially perhaps as regards X-ray injury after the removal of the spleen.

Finally, it may be said that, while it is not likely that the quantitative chemical approach introduced in this study could supersede the well established cytological methods for the study of haemopoietic tissue, it nevertheless must be reckoned as a most useful subsidiary, capable of great development.

SUMMARY

1. The behaviour of acid-insoluble phosphorus and of ascorbic acid and glutathione in rat bone marrow and spleen has been studied under experimental conditions which included dietary changes, X-ray injury, splenectomy, thermal burns and the action of certain myelotoxic substances.

2. A high-fat, carbohydrate-free diet which, although adequate in protein and total calories, failed to support growth, induced profound but not irreversible changes in the content of nucleic acid and lipid phosphorus in bone marrow.

3. The following changes were found in the bone marrow of rats exposed to single X-ray doses ranging from 300 to 800 r. (*a*) The content of nucleic acid phosphorus: immediately after irradiation there were no obvious changes, except perhaps with the highest dose; considerable and progressive breakdown, proportional to the dose, was noticeable a few hours after irradiation, reaching a maximum at 2 days; 7 days after exposure, up to 75 and 50 % recovery was recorded following 300 and 600 r., respectively, but it was much less advanced in the bone marrow of animals which survived 800 r.; at 10 days the pre-irradiation level was regained with the smallest dose, and further marked improvement was observed in those rats which had been irradiated with 500–600 r. (*b*) The content of lipid phosphorus was much less affected than nucleic acid phosphorus, with all doses and at all stages, except at 2 days with 800 r., when little lipid phosphorus remained. (*c*) Ascorbic acid, in a manner analogous to that of nucleic acid phosphorus, decreased in the early, and returned to normal value in the later, post-irradiation period. (*d*) A distinct diminution in the content of glutathione was demonstrated 2 days after irradiation with 500 r., but near-normal values were found at 7 days.

4. The general pattern of post-irradiation changes in the spleen conformed qualitatively to that of the bone marrow, but quantitatively it was much less pronounced.

5. Muscle adenylic acid and yeast adenylic acid, but not inosinic acid, appeared to delay the recovery of nucleic acid phosphorus and ascorbic acid content in the bone marrow and spleen of irradiated animals.

6. The phosphorus distribution of the bone marrow in splenectomized rats examined at various intervals after the removal of the spleen was indistinguishable from that established in unoperated controls; the same was true concerning the response of splenectomized rats towards X-rays.

7. Mild-degree burns of a limited area of skin did not exert any obvious effects on the phosphorus or ascorbic acid content of bone marrow or spleen.

8. Aminopterin and amethopterin selectively affected the bone marrow and had little or no effect upon the spleen. Similar findings were made with colchicine. On the other hand, mustard sulphoxide potentiated by dimethyldithiocarbamate acted, like X-radiation, upon the bone marrow as well as on spleen. Prolonged administration of amidopyrine had no significant effect on rat bone marrow or spleen.

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The Action, *in vitro* and *in vivo*, of some Nitrogen Mustards on Haemolytic Complement

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It has been shown (Boursnell, Francis & Wormall, 1946) that haemolytic complement is rapidly inactivated by small amounts of sulphur mustard (di-2-chloroethyl sulphide; code no. H) and divinyl sulphone. Since the nitrogen mustards have been widely used during recent years in the treatment of some neoplastic diseases, we decided to study the action, *in vitro* and *in vivo*, of a typical nitrogen mustard (di-2-chloroethylmethylamine; code no. HN 2) on the haemolytic complement of man and some other animals.

Haemolytic complement is a complex system containing four functionally distinct components, and it is generally agreed that complement plays an important role in natural immunity and resistance to infection. Although it has not been definitely established that a low blood complement in an animal is associated with a low resistance to infection, many investigators have reported low blood complement titres in some infectious diseases (for the literature see the reviews by Osborn, 1937; Ecker, Seifter & Dozois, 1945; Ecker, 1948). If the therapeutic use of the nitrogen mustards, involving a course of intravenous injections of the drug, were to result in a severe diminution of the blood complement, this loss might have serious ill effects.

A considerable amount of information is available about the separation of the four components of complement, and about their stability and general chemical properties, but the mode of action of this complex system is still unknown. It is a relatively labile system associated largely with certain serum globulins, and the inactivation of any one component, for example by mild oxidation or by denaturation, leads to complete loss of haemolytic, bacteriolytic and bactericidal effects. Pillemer & Ecker (1941*a*) and Heidelberger (1941) have suggested the use of C′1, C′2, C′3 and C′4 as symbols for the 'midpiece', 'endpiece', and third and fourth components respectively, and this nomenclature is used in the present paper.

The main object of the present investigations was to determine whether the clinical use of HN 2 and related nitrogen mustards is likely to lower seriously the blood-complement level of the injected individuals. Our *in vitro* tests soon showed that HN 2, like the sulphur mustard (H), rapidly destroys haemolytic complement, and the investigations were therefore extended to include a more general study of the anticomplementary action of various nitrogen mustards and some related compounds. It was hoped that these experiments would yield useful information about (*a*) the chemical properties of individual components of complement, and (*b*) the nature of the action of nitrogen mustard on proteins and related compounds. Some of the results of these investigations have been reported briefly elsewhere (Watkins & Wormall, 1948). Since the work described here was completed, Southam & Goldsmith (1951) have confirmed our observation that complement is inactivated by nitrogen mustard *in vitro*, and they have also found that no depression of human complement titre occurs following the clinical use of HN 2 and X-rays.

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EXPERIMENTAL

Materials

Di-2-chloroethylmethylamine (code no. HN 2). This was prepared, as the hydrochloride, by the method of Hanby & Rydon (1947).

Di-2-hydroxyethylmethylamine. This compound (Light and Co. Ltd.) was freshly distilled under reduced pressure immediately before use.

NN′-Di-2-chloroethyl-NN′-dimethylpiperazinium chloride. Prepared as described by Hanby & Rydon (1947). Melting point of picrate, 205°.

2-Chloroethyl-2′-hydroxyethylmethylamine. This was isolated via the picrate from a mixture of reaction products, as described by Hanby & Rydon (1947). Melting point of mercurichloride, 197°.

Haemolytic complement

Fresh serum. Normal guinea pig serum was used as the source of complement for most of the experiments on the *in vitro* action of nitrogen mustard on haemolytic complement.

Freeze-dried serum. Human, rabbit and guinea pig sera were dried and kept for use as standards for the experiments on the effect of the injection of di-2-chloroethylmethylamine on the serum haemolytic-complement level in man, rabbit and guinea pig. Samples of bulked serum (0·50 or 1·0 ml. in each tube) were freeze-dried as described by

Greaves (1946) and were sealed under vacuum. The sera were reconstituted in distilled water when required, and were used only on the day on which the tube had been opened. As far as could be judged from a comparison of the complement activity of various standards prepared at different times, no marked loss of activity occurred when the dried standards were kept at room temperature for up to at least 6 months.

Complement lacking C'1 and C'2. Serum was heated at 56° for 30 min. and then diluted with 9 vol. 0·9% (w/v) NaCl.

Complement lacking C'3. Zymosan, the insoluble carbohydrate of yeast used for the specific inactivation of the third component of complement, was prepared by the method of Pillemer & Ecker (1941*b*). The amount of carbohydrate required to inactivate the third component was determined for each sample of serum in a preliminary experiment. The carbohydrate was suspended in distilled water, heated on a boiling-water bath for 30 min. and then centrifuged; the supernatant solution was removed and the residue allowed to cool. Guinea pig serum (1 vol.) was added to this residue and the suspension was kept in a water bath at 37° for 2 hr. with frequent shaking. After the addition of 0·9% NaCl (9 vol.) the suspension was centrifuged and the supernatant solution was separated and its pH was brought to 7·5.

Complement lacking C'4. Complement deficient in the fourth component was prepared by the method of Gordon, Whitehead & Wormall (1926). Undiluted serum (1 ml.) was treated with 0·2 ml. of 0·2 N-NH_3 and the mixture was kept at 37° for 1·25 hr. At the end of this time, 0·16 ml. of 0·2 N-HCl and 8·6 ml. 0·9% NaCl were added.

Separation into 'midpiece' and 'endpiece' fractions. Guinea pig serum (1 vol.) was diluted with CO_2-saturated water (9 vol.) and a slow stream of CO_2 was passed through the mixture which was cooled in ice-water during this process. After 20 min. the mixture was centrifuged and the precipitate ('midpiece' fraction) was washed with 0·02 M-phosphate solution of pH 5·4, and finally dissolved in 10 ml. 0·9% NaCl; the pH of the solution was adjusted to 7·5 with 0·1 M-$NaHCO_3$. The original supernatant solution ('endpiece' fraction) (10 vol.) was treated with 10% (w/v) NaCl (1 vol.) and the CO_2 was removed *in vacuo*; 0·1 M-$NaHCO_3$ was added to bring the pH to 7·5.

Methods

Haemolytic system. Varying amounts of diluted serum, or other mixture being tested for complement activity, were measured into a series of tubes, and 0·9% NaCl was added, where necessary, to bring the total volume to 1·0 ml. in every tube. A 3% suspension (1·0 ml.) of sensitized washed sheep cells was added to each tube and the tubes were immersed in a water bath at 37° for 30 min. Mayer, Osler, Bier & Heidelberger (1946, 1948) have shown that Ca^{++} and Mg^{++} are essential constituents of the haemolytic system, and in experiments involving dialysis or fractionation of the serum we therefore ensured that there were optimum concentrations of these two ions in the final mixtures.

Measurement of complement activity. In the experiments on the *in vitro* action of nitrogen mustard on complement the extent of haemolysis was estimated visually after 10, 20 and 30 min., and was recorded as follows: 4 (complete haemolysis); 3, 2, 1 and tr. (trace) (intermediate degrees of haemolysis); 0 (no haemolysis).

For the determination of the complement level in rabbits, guinea pigs and human subjects before and after the injection of nitrogen mustard, the degree of haemolysis was estimated colorimetrically. After the mixtures had been kept at 37° for 30 min. they were centrifuged and the colour intensity of the liberated haemoglobin in the supernatant solution was measured, after suitable dilution, in a photoelectric colorimeter (Evans Electroselenium Ltd., portable model) using a green Ilford Spectrum filter no. 624. The results were expressed as a percentage of the reading given by a mixture showing complete haemolysis. A graph was then constructed from the values for the percentage haemolysis given by different volumes of serum. The amount of undiluted serum (v) required to produce 50% haemolysis was read off from the curve, and the complement content of the serum was recorded in arbitrary '50% units'/ml. ($1·0/v$). This method is essentially similar to that described by Mayer, Eaton & Heidelberger (1946) and Kent, Bukantz & Rein (1946).

In experiments involving a comparison of the serum complement levels of an animal on different days, the results were expressed as the ratio of the number of 50% units/ml. in the tested serum to the number of 50% units/ml. in the 'standard' freeze-dried serum. When small amounts of serum only were available, or when many titrations had to be carried out on the same day, it was not always possible to titrate the sera over the whole range of partial lysis. For these determinations, therefore, the 50% unit of complement activity was obtained from a single analysis using the conversion factors calculated by Mayer, Osler *et al.* (1946) from the von Krogh equation $x = K(y/(1-y))^{1/n}$ which expresses the S-shaped haemolysis curve.

Since rabbit and human sera frequently contain non-specific haemolysins, these sera were subjected to one absorption with sheep cells before complement determinations were made on them. Packed washed sheep cells (0·2 ml.) were added to the serum (1·0 ml.) and the mixtures were kept at room temperature for 10 min., after which the cells were removed by centrifuging.

RESULTS

In vitro action of nitrogen mustards on haemolytic complement

Effect of HN2 on guinea pig, human and rabbit complement

The action of di-2-chloroethylmethylamine on guinea pig serum complement at 37° and pH about 7·5 has been studied.

The crystalline HN2 hydrochloride was dissolved in 0·9% NaCl and varying amounts of this solution were immediately added to 0·5 ml. samples of guinea pig serum, with 0·9% NaCl to give a total volume of 1·5 ml. in each tube. Phenol red (1 drop of 0·04%) was added to each mixture and each was then kept at 37° with occasional gentle shaking. The pH was maintained at 7·5 by adding dilute NaOH where necessary, and 0·9% NaCl was added to the other tubes to compensate for the volume change. Samples (0·10 ml.) were withdrawn from each mixture at intervals and diluted with 0·9% NaCl (0·90 ml.). The complement activity of each diluted sample was then determined as follows: measured volumes (0·5, 0·2 and 0·1 ml.) were

added to tubes containing 0·5 ml. of a 3% suspension of sensitized sheep cells, 0·9% NaCl was added to give a total volume of 1·0 ml. in each tube, and haemolysis readings were taken after 10, 20 and 30 min. at 37°.

The results showed (Table 1) that partial inactivation of complement occurred in 10 min. when 0·5 ml. of guinea pig serum was treated at 37° with 2·5 mg. of the vesicant, and complete inactivation was effected in 35 min. by 1·25 mg. At room temperature inactivation was less rapid, but the complement activity of 0·5 ml. of guinea pig serum was almost completely destroyed by treatment with 1·0 mg. of HN 2 for 1 hr., and with 10 mg. about half the activity had disappeared within 15 min.

Other experiments showed that the haemolytic complements of human and rabbit sera are similarly inactivated by treatment with HN 2.

Effect, on complement, of 'hydrolysed' HN 2 and of the individual products of this 'hydrolysis'

The nitrogen mustards undergo a series of reactions in aqueous solution (Hanby, Hartley, Powell & Rydon, 1947; Golumbic, Fruton & Bergmann, 1946), and experiments were therefore carried out to determine the complement-inactivating power of 'hydrolysed' nitrogen mustard and some of the individual reaction products. The inactivating power of a 0·25% solution of HN 2 in normal saline which had been kept for 26 hr. at 37° and pH 7·5 was compared with that of a freshly prepared solution of the same strength. The results showed that the ability of the hydrolysed material to inactivate complement was very considerably less than that of an equivalent amount of unhydrolysed HN 2. Only slight inactivation was effected in 180 min. by a volume of the hydrolysed HN 2 solution containing the equivalent of 2·5 mg. of the unhydrolysed amine, whereas comparable inactivation was effected in 60 min. by a tenth of the volume of freshly prepared HN 2 solution.

Table 1. *The action of HN 2 on guinea pig complement at* 37°

HN_2 added to 0·5 ml. of guinea pig serum (mg.)	Volume of diluted sample tested (ml.)	Complement activity* of the samples taken after				
		10 min.	30 min.	60 min.	120 min.	180 min.
0 (control)	0·50	4	4	4	4	4
	0·20	4	4	4	4	4
	0·10	2	2	2	2	1
0·25	0·50	4	4	4	2	2
	0·20	4	3	2	tr.	0
	0·10	2	tr.	0	0	0
1·25	0·50	4	1	0	0	0
	0·20	3	0	0	0	0
	0·10	1	0	0	0	0
2·50	0·50	4	0	0	0	0
	0·20	2	0	0	0	0
	0·10	0	0	0	0	0

* Haemolysis readings taken after 30 min.

In view of the clinical use of the nitrogen mustards and the possibility that a similar inactivation might be produced *in vivo*, tests were made to determine the lowest concentration of HN 2 which would destroy the complement activity of serum. Varying amounts of a freshly made 0·1% solution of HN 2 hydrochloride in saline were added to 0·5 ml. samples of guinea pig serum, and complement determinations were made on the mixtures at intervals. The results showed that the haemolytic-complement activity of the serum was completely destroyed in 6 hr. by treatment at 37° with 0·2 mg. of HN 2, and that appreciable inactivation was brought about in this time by as little as 0·05 mg.

Treatment of haemolysin with amounts of HN 2 appreciably greater than those used to inactivate complement did not decrease the activity of the serum, and exposure of sensitized or unsensitized sheep cells to corresponding amounts of the vesicant did not influence their subsequent haemolysis by complement. The inhibitory effect of nitrogen mustard on the haemolysis of sensitized red cells by complement must, therefore, be due to the destruction or inactivation of one or more of the components of complement.

Further experiments were made to determine how rapidly the power to inactivate complement is lost when aqueous solutions of HN 2 are kept at 37°.

Samples (10 mg.) of HN 2 were dissolved in 0·9% NaCl (2·0 ml.) and the solutions were kept at 37° for varying lengths of time, the pH being maintained at about 7·5. Measured volumes of these solutions of hydrolysed HN 2 were added to guinea pig serum (0·5 ml.), and 0·9% NaCl was added to give a total volume of 1·5 ml. in each tube. The mixtures were kept at 37°, the pH again being maintained at 7·5. Samples were taken from each mixture at intervals and diluted with 9 vol. of 0·9% NaCl. The complement activity of these diluted samples was then measured.

The results (Table 2) showed that hydrolysis for 30 min. slightly reduced the inactivating power of the nitrogen mustard, but even with hydrolysis for 2·5 hr. there was not complete loss of complement-inactivating capacity.

Di-2-hydroxyethylmethylamine in concentrations of up to 125 mg./0·5 ml. guinea pig serum effected no destruction of the complement activity, and this hydrolysis product of di-2-chloroethylmethylamine is apparently not the agent responsible for the complement-inactivating power of hydrolysed nitrogen mustard solutions. Similarly, it was found that the piperazinium dimer, *NN'*-di-2-chloroethyl-*NN'*-dimethylpiperazinium chloride, was without action on complement at concentrations comparable with those of HN2 which rapidly produce inactivation. The chlorohydrin, 2-chloroethyl-2'-hydroxyethylmethylamine, on the other hand, showed some capacity for reducing the complement activity of guinea pig serum, although the inhibition produced was less than that given by a fresh HN2 solution. Thus, a solution containing 2·5 mg. of the chlorohydrin hydrochloride effected complete inactivation of the complement in 0·5 ml. guinea pig serum in 120 min. at 37°, whereas the freshly prepared HN2 solution of the same strength gave almost complete inactivation in 10 min.

Table 2. *The action, on complement, of previously 'hydrolysed' HN2*

'Hydrolysed' HN2 added* (mg.)	Duration of prior hydrolysis of HN2 (min.)	Volume of diluted sample tested (ml.)	Complement activity† of the mixture of serum and hydrolysed HN2 after	
			20 min.	60 min.
1·0	0	0·50	4	0
		0·20	1	0
		0·10	0	0
1·0	30	0·50	4	2
		0·20	2	0
		0·10	0	0
1·0	150	0·50	4	4
		0·20	3	1
		0·10	tr.	0
5·0	0	0·50	0	0
		0·20	0	0
		0·10	0	0
5·0	30	0·50	0	0
		0·20	0	0
		0·10	0	0
5·0	150‡	0·50	3	0
		0·20	0	0
		0·10	0	0
0 (control)	0	0·50	4	4
		0·20	3	3
		0·10	tr.	tr.

* Amount added to 0·5 ml. of guinea pig serum.
† Haemolysis readings taken after 30 min.
‡ Hydrolysed for 45 min. at 20° and a further 105 min. at 37°.

Action of some compounds in protecting complement against inactivation by HN2

HN2 and its half-hydrolysis product, 2-chloroethyl-2'-hydroxyethylmethylamine, both undergo cyclization to form ethyleniminium ions and, since both these substances inhibit complement action, the possibility arises that it is the ethyleniminium ions which are responsible for the inactivation. In order to test this possibility, substances which are known to react with ethyleniminium ions were introduced into the complement-nitrogen mustard system. Table 3 shows the results of an experiment in which 0·1 N-sodium thiosulphate (1 vol.) was mixed with a freshly prepared 0·5 % (w/v) HN2 solution (1 vol.), and the mixture was added at once to guinea pig serum. In the presence of this concentration of thiosulphate the nitrogen mustard failed to inactivate complement. Control tests showed that in these concentrations sodium thiosulphate alone did not affect the haemolytic power of guinea pig serum, showing that this substance is not anti-complementary or lytic *per se*. Treatment with lower concentrations of sodium thiosulphate did not completely prevent inactivation of complement, although the amounts added were in excess of those calculated to 'neutralize' all the

ethyleniminium ions liberated from the HN 2 used.

The results suggest that under these conditions complement and thiosulphate compete for reaction with the ethyleniminium ions. The inactivation of complement by 2-chloroethyl-2′-hydroxyethylmethylamine was similarly prevented by adding sodium thiosulphate. Hexamethylenetetramine and phosphate ions were also found to protect complement against inactivation by nitrogen mustard, although in equimolar concentrations they were not as effective as thiosulphate ions. This is in accordance with the degree of avidity with which hexamethylenetetramine and thiosulphate react with ethyleniminium ions, as shown by Fruton, Stein, Stahmann & Golumbic (1946).

when added to rabbit serum in concentrations up to $1{\cdot}5 \times 10^{-3}$ M, had no effect on the haemolytic-complement activity of the serum, nor did it protect the complement against inactivation by HN 2.

Action of HN 2 on the individual components of complement

Experiments were carried out to determine whether all four components of complement are sensitive to the action of nitrogen mustard or whether the vesicant would prove a useful reagent for the specific inactivation of one or two components. Guinea pig serum samples which had been treated in such a way as to destroy specifically one or two complement components were added to other

Table 3. *The effect of sodium thiosulphate on the inactivation of complement by HN 2*

Solution added* to complement	(ml.)	Volume of diluted sample (1:10) tested (ml.)	Complement activity† of sample taken after 30 min.	60 min.	120 min.
0·25% HN 2 in 0·9% NaCl	0·1	0·5	4	3	2
		0·2	2	1	0
		0·1	0	0	0
	0·5	0·5	1	0	0
		0·2	0	0	0
		0·1	0	0	0
	1·0	0·5	0	0	0
		0·2	0	0	0
		0·1	0	0	0
0·25% HN 2 in 0·05 N-$Na_2S_2O_3$	0·1	0·5	4	4	4
		0·2	2	2	2
		0·1	tr.	tr.	0
	0·5	0·5	4	4	4
		0·2	2	2	2
		0·1	tr.	0	0
	1·0	0·5	4	4	4
		0·2	2	2	2
		0·1	tr.	0	0
Control (untreated complement)	—	0·5	4	4	4
		0·2	2	2	2
		0·1	tr.	0	0

* To 0·5 ml. guinea pig serum.

† Haemolysis readings after 30 min.

In view of the reaction of HN 2 with the α-amino groups of amino-acids (Fruton, Stein & Bergmann, 1946), glycine was tested for its capacity to prevent the inactivation of complement by the vesicant. The results showed, however, that glycine has no protective action in this respect.

The inhibition, by nitrogen mustard, of enzymes concerned with choline metabolism has been attributed (Barron, Bartlett & Miller, 1948) to the similarity in structure between choline and the ethyleniminium ions formed by the nitrogen mustards. We decided, therefore, to determine whether choline has any action on complement, and whether it can protect complement against inactivation by HN 2. It was found that choline,

samples which had been treated with HN 2, and the mixtures were tested for full complement activity.

Typical results of an experiment in which ammonia-treated serum (lacking C′ 4) and heated serum (lacking C′ 1 and C′ 2) were added to serum which had been inactivated by treatment with 2 vol. of an 0·25% (HN 2) solution for 35 min. at 37° are shown in Table 4. Partial reactivation occurred when the ammonia-treated serum was added to the HN 2-treated serum, but there was no reactivation when heated guinea pig serum was added to the latter. This suggests that the fourth component (C′ 4) is less rapidly inactivated by HN 2 than one or both of the heat-labile com-

ponents C′1 and C′2. Further experiments showed that longer treatment with HN2 gave an inactive serum which was not activated by the addition of ammonia-treated serum (deficient in C′4).

Other experiments showed that the addition of serum lacking complement component C′3 (i.e. guinea pig serum treated with an insoluble carbohydrate of yeast) to HN2-inactivated complement (guinea pig serum treated with 1 vol. of 0·25 % HN2 at 37°) gave a mixture with significant complement activity, if the treatment with HN2 was short (e.g. 15–30 min.). Longer treatment with HN2 under these conditions resulted in complete loss of C′3 activity.

of nitrogen mustard-inactivated complement could be restored by treatment with this reagent. Samples (0·2 ml.) of guinea pig serum which had been treated with an equal volume of a 0·25 % HN2 solution for various lengths of time were mixed with 0·1N-sodium thiosulphate (0·2 ml.) and the mixtures were tested for complement activity after they had been kept for 10, 30 and 60 min. at room temperature. The results showed that the sodium thiosulphate had effected no reactivation of the HN2-inactivated complement.

It has been suggested (Fruton, Stein & Bergmann, 1946; Loveless & Revell, 1949) that the nitrogen mustards react with proteins by esterification of

Table 4. *Attempted reactivation of HN2-inactivated complement with* (*a*) NH_3*-treated* (*lacking C′4*) *and* (*b*) *heated serum* (*lacking C′1 and C′2*)

(For experimental details see pp. 366 and 369. The HN2-treated serum, NH_3-treated serum and heated serum used in these tests corresponded, respectively, to 1:5, 1:10 and 1:10 dilutions of the original guinea pig serum. Where necessary, 0·9 % NaCl was added to make a total volume of 1·0 ml. for each mixture, and 0·5 ml. of a 3 % suspension of sensitized sheep erythrocytes was then added to each tube.)

HN2-treated serum (ml.)	NH_3-treated serum (ml.)	Heated serum (ml.)	Complement activity of the mixtures* (*a*)	(*b*)
0·5	—	—	0	0
0·5	0·2	—	0	1
0·5	0·5	—	tr.	3
0·5	—	0·2	0	0
0·5	—	0·5	0	0
0·2	—	—	0	0
0·2	0·2	—	0	1
0·2	0·5	—	0	3
0·2	—	0·2	0	0
0·2	—	0·5	0	0
—	0·2	0·2	2	4

* Haemolysis readings after (*a*) 10 min. (*b*) 30 min.

Several experiments were made to determine whether HN2-inactivated complement can be activated by adding 'midpiece' or 'endpiece' fractions of complement. It was found that when the HN2 was allowed to act for only a short time, the addition of 'endpiece', but not 'midpiece', to the HN2-inactivated serum gave a mixture with complement activity. In a few experiments the difference in the degree of reactivation effected by the two fractions was not very marked, but where a difference was observed, it was always in the same direction. An experiment designed to determine the comparative rates of inactivation of the four components by HN2 indicated that C′2 is the component which is most rapidly destroyed, and that C′1, C′3 and C′4 disappear at approximately the same rate.

Attempted reactivation of complement inactivated by HN2

In view of the strong affinity of sodium thiosulphate for ethyleniminium ions, experiments were carried out to determine whether the activity

carboxyl groups. We therefore attempted to regenerate the complement activity of HN2-inactivated serum by treatment with an esterase. A preparation obtained from rat liver was found to be without haemolytic action on sensitized sheep cells and to have no marked destructive action on complement at dilutions at which it was still actively hydrolytic for ethyl butyrate. Treatment of HN2-inactivated rabbit serum with the esterase preparation for 1 hr. did not restore complement activity. The sole object of this experiment was to determine whether the rat-liver enzyme preparation could reverse the inactivation of complement effected by HN2, and the result does not militate against the view that the carboxyl groups of the complement proteins are esterified by the drug.

There is evidence that some of the linkages by which nitrogen mustard is bound to proteins are unstable, and it seemed possible that nitrogen mustard residues might be removed from nitrogen mustard complexes by dialysis or by treatment with very dilute alkali. HN2-inactivated serum was

dialysed against 0·9 % sodium chloride, and in other experiments the inactivated serum was treated with a weakly alkaline buffer (pH 9·0), in the presence of 10 % sodium chloride (to protect complement against inactivation by alkali (Pillemer & Ecker, 1941*c*)), and subsequently dialysed. In all these experiments there was no restoration of the complement activity.

The activity of complement which has been destroyed by oxidation can in certain instances be regenerated by reduction, and it has been suggested (Ecker, Pillemer, Martiensen & Wertheimer, 1938) that inactivation by oxidizing agents might be due to the conversion of thiol groups to disulphide groups. The nitrogen mustards react with sulphydryl groups *in vitro* and experiments were therefore carried out to determine whether reducing agents could reactivate complement which had been inactivated by HN 2. No reactivation occurred when ascorbic acid or potassium cyanide was allowed to act on the inactivated guinea pig serum at pH 7·4 for various lengths of time.

Role of sulphydryl groups in complement inactivation by HN 2

The failure of ascorbic acid or potassium cyanide to restore complement activity to HN 2-treated sera indicates that the inhibition is not due solely to a reversible oxidation of SH groups, but this evidence does not exclude inactivation due to an irreversible reaction with these functional groups. It has been shown (Ecker *et al.* 1938) that sera treated with cuprous oxide, which reacts specifically with SH groups to form mercaptides, lose their capacity to haemolyse sensitized cells, but subsequent addition of dilute potassium cyanide or hydrogen sulphide completely restores complement activity. In the following experiment serum was treated with cuprous oxide to protect the sulphydryl groups, allowed to react with HN 2 and then an attempt was made to reactivate the serum with potassium cyanide.

Cuprous oxide (15 mg.) was added to 3 ml. of dilute guinea pig serum (1 vol. of serum plus 9 vol. of 0·9 % NaCl), and the mixture was shaken gently for 5 min. and then allowed to stand for 45 min. at room temperature; the suspension was then centrifuged to remove the solid. A freshly prepared 1 % HN 2 solution (0·2 ml.) was added to the Cu_2O-treated serum (1 ml.) and also to a sample of untreated guinea pig serum (0·1 + 0·9 ml. of 0·9 % NaCl). The mixtures were kept for 30 min. at 37°, and 0·1 N-$Na_2S_2O_3$ (0·2 ml.) was then added to each mixture to prevent further reaction of the nitrogen mustard with complement. After a further 5 min. at 37° a sample (0·5 ml.) of each solution was added to 0·01 M-KCN (0·25 ml.); the mixtures were kept for 15 min. at room temperature and 0·5 ml. of a sensitized sheep-cell suspension (0·5 ml.) was added to each tube, and the extent of haemolysis after 10 and 30 min. was noted.

Table 5. *Attempt to reactivate, with* KCN, *guinea pig serum complement inactivated with* Cu_2O *and subsequently treated with HN 2*

(The volumes given in this table refer to ml. of serum diluted 1:10. Where necessary, 0·9 % NaCl was added to bring the volume of the mixture to 0·75 ml. before the sensitized red cells were added.)

Untreated guinea pig serum (ml.)	HN 2-treated serum (ml.)	Cu_2O-treated serum (ml.)	Cu_2O- and HN 2-treated serum (ml.)	KCN (0·01 M) (ml.)	Complement activity of the mixture* (*a*)	(*b*)
0·5	—	—	—	—	4	4
0·5	—	—	—	0·25	4	4
—	0·5	—	—	—	0	tr.
—	0·5	—	—	0·25	0	tr.
—	—	0·5	—	—	0	0
—	—	0·5	—	0·25	2	4
—	—	—	0·5	—	0	0
—	—	—	0·5	0·25	0	tr.

* Haemolysis readings after (*a*) 10 min. (*b*) 30 min.

The results (Table 5) show that complement inactivated with cuprous oxide and subsequently treated with HN 2 is not reactivated by potassium cyanide. The fact that HN 2 inactivates complement even when the sulphydryl groups of the latter have been protected by cuprous oxide indicates that complement groups other than sulphydryl are concerned in the inactivation by HN 2.

Effect, on complement, of various nitrogen mustards and related compounds

Aromatic nitrogen mustards. In view of the interesting observations of Haddow, Kon & Ross (1948) on the cytotoxic action of a series of aromatic nitrogen mustards, we decided to examine the complement-inactivating power of typical members of this series. Samples of the following compounds (four aromatic nitrogen mustards and one related compound with a single 2-chloroethyl side chain) were kindly supplied by Dr Ross; *NN'*-di-(2-chloroethyl)-*p*-anisidine, *NN'*-di-(2-chloroethyl)-*p*-toluidine, *NN'*-di-2-chloroethyl-2-naphthylamine, *p*-chloro-*NN'*-di-(2-chloroethyl)aniline and *N*-ethyl-*N*-2-chloroethylaniline.

Guinea pig serum (0·2 ml.) was added to 10 mg. samples of each of these compounds, and the solid was ground with a glass rod. The mixtures were kept for 2 hr. at 37°, and then centrifuged to remove the sparingly soluble aromatic nitrogen mustards. The complement activity of the supernatant solutions was then determined.

Under the conditions of these experiments the aromatic nitrogen mustards tested were without action on complement. These compounds have a very low solubility in water and consequently a very much slower rate of hydrolysis than the aliphatic nitrogen mustards, but even when kept for 21 hr. at 37° aqueous suspensions gave supernatant solutions devoid of complement-inactivating capacity.

2-Chloroethyldimethylamine. Since compounds with a single 2-chloroethyl side chain are ineffective as tumour-inhibitory or carcinogenic agents, we decided to determine whether complement inactivation could be effected by a monofunctional nitrogen mustard.

Varying amounts (0·1–0·3 ml.) of an 0·02M solution of 2-chloroethyldimethylamine were added to samples (0·3 ml.) of fresh guinea pig serum, and the mixtures were kept at 37°. Tests made on samples withdrawn at intervals up to 2 hr. showed that no inactivation of complement had occurred; control tests showed that the complement activity of 0·3 ml. of serum was completely destroyed in 30 min. at 37° by 0·3 ml. of 0·02M-HN2 and partially destroyed by 0·1 ml. The experiment was repeated using 0·2M-2-chloroethyldimethylamine, but this larger amount gave only slight inactivation after 3 hr. at 37°.

Diepoxides. In view of the similarity in the cytological effects of the nitrogen mustards and diepoxides (Loveless & Revell, 1949) we decided to examine typical diepoxides (kindly provided by Dr Ross) for their action on complement. Treatment of guinea pig serum (0·5 ml.) with different volumes (0·1–1·0 ml.) of 0·02M solutions of di-2:3-epoxypropyl ether, 1:2:3:4-diepoxybutane and 1:2:5:6-diepoxydiallyl for periods up to 2 hr. failed to inactivate complement, but when the experiments were repeated using 0·5M solutions of these diepoxides, there was considerable diminution in complement activity. The results obtained with 1:2:5:6-diepoxydiallyl are given in Table 6. Solutions (0·5M) of the diepoxides which had been kept for 20 hr. at 37° still retained some capacity to inactivate complement. The results of tests in which complement fractions lacking one or more components were added to serum which had been inactivated by treatment with 1:2:3:4-diepoxybutane indicated that the diepoxides have no selective action on any one component of complement.

Table 6. *The action of* 1:2:5:6-*diepoxydiallyl on guinea pig complement*

1:2:5:6-Diepoxydiallyl added* (mg.)	Volume of diluted† sample tested (ml.)	Complement activity of samples taken after							
		15 min.		30 min.		60 min.		120 min.	
—	0·5	4	4	4	4	4	4	4	4
	0·2	2	4	2	4	2	3	1	3
	0·1	0	2	0	2	0	2	0	tr.
5·7	0·5	4	4	4	4	4	4	3	4
	0·2	2	4	2	3	1	3	0	3
	0·1	0	2	0	1	0	0	0	0
28·5	0·5	4	4	3	4	3	4	0	1
	0·2	2	3	tr.	3	0	2	0	0
	0·1	0	tr.	0	0	0	0	0	0
57·0	0·5	4	4	2	4	tr.	2	0	0
	0·2	1	3	0	2	0	0	0	0
	0·1	0	0	0	0	0	0	0	0
Haemolysis readings after (min.)		10	30	10	30	10	30	10	30

* To 0·5 ml. guinea pig serum.
† Equivalent to a 30-fold dilution of the original serum.

The effect of the injection of HN2 on the serum haemolytic-complement activity in man, rabbit and guinea pig

Experiments on guinea pigs

HN2 hydrochloride (5 mg.) was dissolved in sterile saline (5 ml.) immediately before use. The solution was injected into the heart, except in the case of guinea pig no. 2 which received an intravenous injection into the ear. Blood was taken from each of the eight guinea pigs before and at a suitable interval (1–6 hr.) after the injection. The samples were obtained by pricking an ear vein with a needle and squeezing out drops of blood which were collected in a small tube ($2 \times \frac{1}{4}$ in.). About 0·2 ml. of serum was obtained by this method, and the complement activities of these samples were determined and expressed in 50% units/ml.

The results (Table 7) showed that the intravascular injection of HN2, in amounts of 1·33–2·86 mg./kg. of body weight, had no marked effect on the haemolytic-complement activity of the serum of the injected guinea pigs during a period of 1–6 hr. after the injection.

Experiments on rabbits

Five rabbits were given intravenous injections of HN2 (a 0·1% solution of the hydrochloride in sterile 0·9% NaCl), and the doses given ranged from 0·5 to 2·0 mg./kg. body weight. One rabbit received one injection only, three had two injections and one had three injections, with intervals of

Table 7. *Complement activity of serum of guinea pigs before and after injection with HN2*

Guinea pig no.	HN2 injected (mg./kg. body wt.)	Blood sample taken	Complement activity (50% units/ml.)
1	2·66	Before injection	129
		1 hr. 20 min. after injection	144
2*	1·82	Before injection	130
		2 hr. 50 min. after injection	130
3	1·33	Before injection	110
		1 hr. 15 min. after injection	116
4	2·86	Before injection	135
		2 hr. 30 min. after injection	126
5	2·22	Before injection	145
		4 hr. after injection	136
6	2·15	Before injection	142
		4 hr. after injection	136
7	1·54	Before injection	110
		1 hr. after injection	143
		6 hr. after injection	156
8	2·44	Before injection	185
		1 hr. 15 min. after injection	185
		6 hr. after injection	196

* The HN2 was injected intravenously into this guinea pig; the other animals received intracardiac injections.

Table 8. *Complement activity of serum of rabbits before and after injection with HN2*

Rabbit no.	HN2 injected (mg./kg. body wt.)	Blood sample taken	Complement activity (50% units/ml.)
506	0·5	Before injection	12·5
		20 min. after injection	11·1
722	0·5	Before injection	9·1
		20 min. after injection	8·4
506*	0·5	Before injection	10·0
		1 hr. after injection	8·4
722*	0·5	Before injection	6·7
		1 hr. after injection	7·1
723	0·5	Before injection	12·5
		1 hr. 30 min. after injection	12·5
510	0·5	Before injection	20·0
		1 hr. 30 min. after injection	16·7
723*	1·0	Before injection	7·4
		3 hr. 40 min. after injection	7·2
510*	1·0	Before injection	12·1
		3 hr. 40 min. after injection	11·5
723*	2·0	Before injection	6·7
		2 hr. 20 min. after injection	7·1
		5 hr. after injection	7·1
		24 hr. after injection	7·7
767	2·0	Before injection	6·9
		2 hr. 20 min. after injection	6·9
		5 hr. after injection	6·9
		24 hr. after injection	7·4

* 1 week after the previous injection.

1 week between the injections. Blood samples were taken from each rabbit, from the ear not used for the injection, before and at various intervals after the injection. The complement activities of the sera were determined and recorded in terms of 50% units/ml. Parallel titrations were also made with a freeze-dried bulked rabbit serum which was kept as the 'standard' for all the tests in this series, and in this way it was possible to compare the serum complement activity on any one day with the activities recorded for the same rabbits at earlier and later stages of the experiments. The rabbits injected with the largest amount of HN2 (2·0 mg./kg. body weight) showed some loss of appetite about 6 days after the injection.

The results of these experiments (Table 8) showed that there was no consistent change in the complement titre of the blood 20 min.–24 hr. after the injection of relatively large amounts of HN2; the changes observed were within the normal variation in serum complement level. Furthermore, the values for the ratio of the complement activity of the tested serum to the activity of the 'standard' dried rabbit serum showed that the serum complement activity 6 or 7 days after the injection, even with a dose as large as 2·0 mg. of HN2/kg. body weight, was not significantly lower than the level before the injection.

Observations on man

Serum samples from patients receiving intravenous injections of HN2 during the period December 1948–June 1949 were examined for complement activity. The patients normally received four injections of 0·1 mg. HN2/kg. body weight, given daily or every other day. Blood was taken by venepuncture immediately before the first injection and on the day following the final injection. Complement titrations were carried out on a 'standard' freeze-dried preparation of human serum, in parallel with the titrations on the patient's serum. A typical pair of curves for a patient's serum (case no. 4, before injection) and the reconstituted 'standard' serum, are given in Fig. 1; the dotted lines indicate the amount of serum required for 50% haemolysis.

The complement activities of the serum samples taken from 18 patients, before and after injection, are summarized in Table 9; the age and disease of the patients are included where this information is available. These results show that there is no general fall in the serum complement activity of man following a course of nitrogen mustard injections customarily given for the treatment of certain neoplastic and related conditions. The blood complement activity of man varies appreciably from day to day, and the variations cannot be attributed solely to variations in the haemolytic system used in the tests, as shown by the typical results recorded in April–June 1949, for five normal individuals (Table 10). The variations observed with the subjects injected with HN2 were thus not outside the range of variation for subjects who have not been injected with the drug.

DISCUSSION

The nitrogen mustards have important biological effects, some of which can be attributed to reactions of the drug with proteins and related compounds. HN2, a typical nitrogen mustard, reacts with amino, carboxyl, sulphydryl and sulphide groups of amino-acids and peptides, and it inactivates several enzymes (see, for example, Fruton, Stein & Bergmann (1946); Dixon & Needham (1946); Thompson (1947); for other references see Boyland (1949); Philips (1950); Karnofsky (1950)). Quantitative information about the reaction of HN2 with proteins at pH 7·5 has been obtained more recently with the aid of the ^{15}N-labelled drug, and it has been found that part of the HN2 which is bound to proteins is very firmly attached (Burnop, Richards, Watkins & Wormall, 1951).

The inactivating action of HN2 on haemolytic complement reported here can most readily be explained by assuming that the drug reacts with essential groups in the complement system. The nitrogen mustard proteins produced are not *per se* anti-complementary, and we have evidence from our immunological investigations (Watkins & Wormall, 1952) and other studies with HN2, that even if the drug effects denaturation of proteins this change is not the cause of the altered immunological specificity resulting from the action of HN2 on proteins. As yet, however, it is not possible to relate the change in immunological specificity, or the inactivation of haemolytic complement, or the inactivation of certain enzymes, to specific changes in functional protein groups.

Since complement is readily inactivated by low concentrations of HN2 at 37° and pH 7·5, the possibility of a serious depletion of the blood complement following intravenous injection of the drug into man could not be dismissed without further investigation. The doses usually given to man (a series of four or five intravenous injections, each of about 0·1 mg. of HN2 hydrochloride/kg. of body weight) will give considerably lower concentrations of HN2 in the plasma than are required to completely inactivate complement *in vitro*, though significant destruction of complement might be expected when larger doses are injected (e.g. up to 1·0 mg./kg. over a period of 4–7 days, or a single injection of 0·4 mg./kg.). Furthermore, there is the possibility that the anti-complementary action of HN2 might continue for a long time after the injection of the drug, for we have found that 'hydrolysed' HN2 possesses significant complement-inactivating power; there is also the possibility that HN2 may have a cumulative anti-complementary effect, since it is cumulative in relation to toxicity (Karnofsky, 1950).

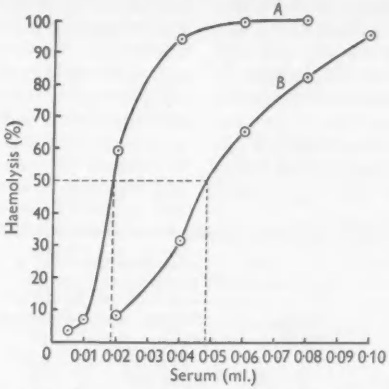

Fig. 1. Complement titration curves for human sera. *A*, serum from patient no. 4 before injection with HN2; *B*, standard dried human serum.

Table 9. *Complement activity of serum samples from human subjects before and after injection with HN2*

Case no.	Age (yr.)	Disease	Serum sample	Complement activity of serum (50% units/ml.) Subject (*X*)	Standard (*Y*)	Ratio (*X*/*Y*)
1	32	Mikulicz's syndrome	Before injection	38·5	18·5	2·1
			After injection	45·4	13·5	3·4
2	50	Hodgkin's disease	Before injection	76·9	27·0	2·8
			After injection	55·5	16·7	3·4
3	66		Before injection	47·0	17·0	2·8
			After injection	55·5	17·0	3·3
4	42	Reticulosis	Before injection	55·5	19·6	2·8
			After injection	66·5	21·3	3·1
5	28	Hodgkin's disease	Before injection	83·4	27·0	3·1
			After injection	66·7	21·3	3·1
6	55	Carcinomatosis	Before injection	66·7	21·3	3·1
			After injection	62·7	20·4	3·1
7	42		Before injection	43·4	10·6	4·1
			After injection	77·0	17·2	4·5
8			Before injection	23·8	10·6	2·2
			After injection	55·5	17·5	3·2
9	31	Lymphadenoma	Before injection	45·5	12·8	3·5
			After injection	59·0	15·6	3·8
10	54	Carcinoma	Before injection	47·6	7·0	6·8
			After injection	50·0	6·7	7·4
11	27	Hodgkin's disease	Before injection	76·9	7·0	10·9
			After injection	66·7	10·6	6·3
12	22	Hodgkin's disease	Before injection	50·0	14·3	3·5
			After injection	83·4	18·5	4·5
13	47	Lymphadenoma	Before injection	37·0	9·8	3·8
			After injection	55·5	16·1	3·5
14	46	Lymphatic leukaemia	Before injection	62·5	9·6	6·5
			After injection	66·7	10·9	6·1
15	37	Lymphadenoma	Before injection	47·6	9·6	5·0
			After injection	52·6	10·9	4·8
16	66		Before injection	52·6	12·3	4·3
			After injection	50·0	10·9	4·6
17	13	Sarcoma	Before injection	43·5	9·6	4·5
			After injection	55·5	14·5	3·8
18			Before injection	62·5	12·1	5·2
			After injection	66·7	11·0	6·1

The results of our investigations have shown, however, that the nitrogen mustard therapy normally adopted does not involve any risk of a significant destruction of the blood complement. It seems probable that the drug combines with various blood and other tissue proteins and that the proteins of complement are not selectively attacked. In other investigations we have found that the risk of producing hypersensitivity to HN 2 by repeated injections of the drug is not serious (Watkins & Wormall, 1952), but in this case the chief factor concerned is probably the lability of many of the linkages between the nitrogen mustard and the body proteins.

Part of the HN 2 which is readily bound by proteins at pH 7·5 can be removed by dialysis (Burnop *et al.* 1951), but dialysis does not restore the activity of HN 2-inactivated complement. All our efforts to reverse the inactivating effect of HN 2 on the whole complement system have been unsuccessful, but we have not yet explored the possibility of reversing the effect of the drug on the individual separated components. This inactivation of complement is apparently of a fairly specific character, for amounts of HN 2 considerably greater than that required to inactivate complement have no detectable destructive action on the erythrocyte-sensitizing antibody of haemolytic serum.

The diepoxides, which have been used for effecting cross linking between carboxyl groups of wool fibres and which have cytological effects similar to those produced by the nitrogen mustards, are much less powerful inactivators of complement than is HN 2. For the destruction of complement the diepoxides we have tested have to be used in molar concentrations about 25 times that required in the case of HN 2. However, this difference does not necessarily indicate a difference in the mode of complement inactivation by the two groups of compound, since the chemical reactivity of the diepoxides is considerably less than that of HN 2. Thus the inactivation of complement by HN 2, or by the diepoxides, may be associated with a cross-linking mechanism of the type suggested by Goldacre, Loveless & Ross (1949) in respect to the production of chromosome abnormalities by the nitrogen mustards.

Table 10. *Complement activity of serum samples taken at weekly intervals from normal subjects*

Subject	Serum sample	Complement activity of serum (50% units/ml.) Subject (X)	Standard (Y)	Ratio (X/Y)
A.W.	1	38·4	10·9	3·5
	2	38·4	12·5	3·1
	3	41·7	10·0	4·2
H.G.R.	1	25·6	10·9	2·4
	2	43·6	12·5	3·5
	3	38·5	10·0	3·9
W.M.	1	30·3	10·9	2·9
	2	33·3	11·5	2·9
	3	27·8	10·0	2·8
R.L.F.T.	1	38·4	10·9	3·5
	2	25·6	12·5	2·0
	3	41·7	10·0	4·2
W.M.W.	1	27·8	12·8	2·2
	2	41·7	9·9	4·2
	3	29·4	10·0	2·9

SUMMARY

1. Haemolytic complement is rapidly inactivated *in vitro* by the nitrogen mustard di-2-chloroethylmethylamine (HN 2), at 37° and pH 7·5. All four components of complement are inactivated.

2. When aqueous solutions of HN 2 are kept, most of the complement-inactivating capacity is lost. Of the 'hydrolysis' products tested, only 2-chloroethyl-2′-hydroxyethylmethylamine showed complement-inactivating power, and this was low compared with that of HN 2.

3. Thiosulphate protects complement against inactivation by HN 2 or by 2-chloroethyl-2′-hydroxyethylmethylamine. Less marked protection is afforded by phosphate and hexamethylenetetramine. On this evidence it is suggested that complement inactivation by HN 2 is due to ethyleniminium ions.

4. The nature of the inactivation has not been established, but reaction of the HN 2 with sulphydryl groups of complement is not the sole cause of inactivation. Attempts to reverse the inactivation effected by HN 2 have failed.

5. Three diepoxides known to effect cross linking of protein molecules inactivate complement, though they are much less effective in this respect than is HN 2. These results, and others with a

monofunctional nitrogen mustard, are not inconsistent with the view that cross linking between protein molecules is an important factor in complement inactivation by the nitrogen mustards.

6. Five aromatic nitrogen mustards tested had no destructive action on complement, possibly because of their low solubility in water.

7. The intracardiac or intravenous injection of HN 2 into guinea pigs or intravenous injection into rabbits (single injections of 0·5–2·8 mg./kg. body weight) caused no significant fall in the serum haemolytic-complement activity of these animals.

8. The serum complement activity of 18 patients receiving nitrogen mustard therapy has been studied before and after the course of intravenous injections of HN 2 (four injections, each of 0·1 mg./kg. body weight, over a period of 4–8 days). This drug treatment caused no depression of the serum complement activity.

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The Metabolism of Glucuronic Acid by Xylose-fermenting Coliform Bacteria

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Although the ability of coliform bacteria to ferment D-xylose and L-arabinose is well known, there is relatively little information concerning their ability to ferment the related homomorphous uronic acids, D-glucuronic and D-galacturonic acids.

Kay (1926) found that twenty-one members of the coli-typhosus group were able to utilize glucuronic acid as a carbon source. Some cultures studied further were found to ferment glucuronic acid to form qualitatively the same end products as from glucose. However, Kay pointed out that the lactic and succinic acids found may have arisen from the peptone in the medium. Quick & Kahn (1929) extended this work and in surveying a large number of colon typhoid-dysentery bacteria, found that most strains were capable of fermenting glucuronic acid to form some unspecified acidic products.

It has frequently been observed (cf. Hirst, 1949) that in natural products, such as xylans, both glucuronic acid and xylose occur together, as part of the same molecule, and this has led to the suggestion that xylose may arise by the decarboxylation of glucuronic acid. Indeed, in 1902, Salkowski & Neuberg claimed to have isolated xylose as a product of the decarboxylation of glucuronic acid by bacteria in a putrefying mince. It has, however, been demonstrated (Franken, 1932) that glucuronic and galacturonic acids can be decarboxylated to the homologous pentoses by heating with sulphuric acid. In a recent examination of the hypothesis, Cohen (1949) studied the ability of the K-12 strain of *Escherichia coli* to ferment certain uronic acids and their related pentoses, and showed that cells grown on the one type of substrate would not immediately ferment the other type of substrate. From this, the conclusion was drawn that uronic acids were not metabolized by an initial decarboxylation.

In a previous communication (Heald, 1952) it was shown that the products of the fermentation of xylose and glucuronic acid by certain strains of coliform bacteria were not at all dissimilar, but differed from the fermentation products of glucose. This suggested that glucuronic acid and xylose might be metabolized by the same pathway, which might differ in some respects from the pathway for the anaerobic metabolism of glucose. As a result of experiments carried out to test this hypothesis, it has been found that cultures grown on xylose or glucose were able to adapt to the utilization of glucuronic and galacturonic acids in resting suspension, under anaerobic conditions, and in the absence of fixed exogenous nitrogen. Further it was found that the rate at which adaptation occurred was markedly stimulated by the presence of a readily metabolized second substrate, by a factor (or factors) which could be washed from the cells, or by adenosinetriphosphate (ATP).

MATERIALS AND METHODS

Cultures. The principal cultures used have already been described (Heald, 1952). After the general results described below had been established with five cultures, the remaining work was carried out with four strains of culture 14. These were obtained by plating the culture on to a basal medium agar containing 0·5% xylose and selecting four colonies. These strains were indistinguishable by their fermentation reactions, but were used as separate strains in order to confirm each set of data obtained. At the end of the experiments a comparison of the cultural and general morphological characteristics of these strains, with similar characteristics of the original freeze-dried stock cultures, failed to show that any change had taken place. *Escherichia coli* N.C.T.C. no. 86 and *Aerobacter aerogenes* N.C.T.C. no. 8197 were obtained from the National Collection of Type Cultures.

Culture medium. The medium originally described (Heald, 1952) was modified in order to obtain reproducible growth. The rumen-liquor supplement was replaced by 0·06% Bacto-Tryptone (Difco Laboratories, Detroit, Michigan) and the concentrations of $NaHCO_3$ and acetate were reduced by half. All the cultures grew well on this medium, with 0·4% of the required carbohydrate, but did not grow at all on the medium alone, in 24 hr. at 40°.

Preparation of cells for experiments. Except where stated below, the cells were obtained by inoculating 0·1 ml. of a 24 hr. culture into the liquid basal medium containing 0·4% of the required carbohydrate and incubating at 40° for 16–20 hr. The suspension was centrifuged and the cells were washed twice in bicarbonate saline buffer (Krebs & Henseleit, 1932) saturated with 95% N_2–5% CO_2 at pH 7·2 (Umbreit, Burris & Stauffer, 1945). The suspensions were diluted with buffer to roughly the same density, and suitable quantities were taken for manometric experiments.

Manometry. Acid production was measured in bicarbonate-saline, in Warburg manometers, at 40° under an atmosphere of 95% N_2–5% CO_2. In experiments requiring two substrates, vessels with two side arms were employed.

Anaerobiosis was obtained by placing a small stick of scraped yellow phosphorus in the centre cup.

It was shown that when gas evolution ceased, all the substrates had been utilized (Table 1), and consequently the rate of gas production was taken as a measure of substrate utilization.

Rates are calculated as $q^{Na}_{CO_2}$, (μl. CO_2/0·1 mg. N/hr.).

Table 1. *Quantities of xylose and glucuronate remaining in solution after the initial rapid gas evolution had ceased in fermentation by washed suspensions of coliform bacteria*

Substrate	Amount originally taken (μg.)	Amount found (μg.)	Amount used (μg.)	Amount used (%)
Xylose	688·5	32·5	656·0	95·5
	687·5	29·5	658·0	95·0
Glucuronate	605·0	49·5	555·5	92·0
	612·0	49·5	562·5	91·8

Analytical methods. Carbohydrates were estimated by the method of Hagedorn & Jensen (1923) in solutions from the manometers after first inhibiting further bacterial action by tipping in acid from a second side arm at the end of the experiment. The solution was then brought to pH 7·0 with NaOH and the bacteria were removed by precipitation with $ZnSO_4$ and NaOH (Neish, 1946).

Nitrogen was estimated in samples of the cell suspensions and in other liquids by the method of Chibnall, Rees & Williams (1943).

Substrates. D-Glucose (British Drug Houses Ltd., 'Analar' Dextrose); D-galactose, D-xylose (T. Kerfoot Ltd.); L-arabinose, D-galacturonic acid (L. Light and Co. Ltd.). D-Glucurone was given by Dr G. A. Levvy, a gift to him from Corn Products Co., Ltd. It was converted to the Na salt of the acid by electrometric titration with NaOH to a stable pH of 8·0. At this pH the acid is most probably in the pyranose form (Challinor, Haworth & Hirst, 1931; Smith, 1939) and is therefore comparable to D-galacturonic acid, D-galactose, D-glucose, D-xylose, and L-arabinose. α- and β-glucuronic acid-1-phosphates were given by Mr C. A. Marsh. The sample of β-acid contained 30% of the phosphate, the remainder being most probably mainly potassium phosphate and a small amount of β-glucose-1-phosphate. Glucose and glucuronic acid were absent. ATP was used as the sodium salt, prepared from a sample of the dibarium salt given by Mrs K. Heald.

For use as substrates for growth, the required carbohydrates were sterilized in 25% aqueous solution, either by autoclaving at 15 lb. pressure for 15 min. or, in the case of the sugar acids, by Seitz filtration. In the manometric experiments, the sugars were used as 0·01 M solutions in bicarbonate saline.

RESULTS

The result of shaking cells grown on xylose with glucuronic and galacturonic acids, glucose, galactose xylose, arabinose and α- and β-glucuronic acid-1-phosphates is shown in Fig. 1. Glucose and xylose were fermented immediately. Galactose and α- and β-glucuronic acid-1-phosphates were not fermented

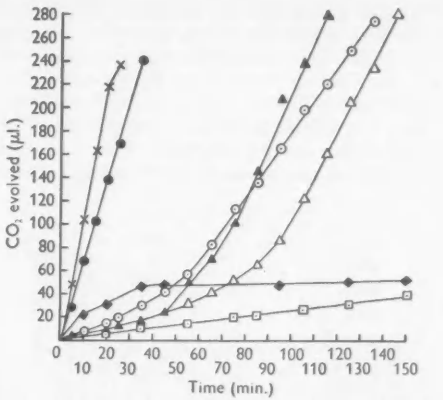

Fig. 1. Acid production from various substrates by cells previously grown on xylose. Flasks contained 153 μg. cell N in 2·25 ml. of bicarbonate saline. Side arms contained 0·5 ml. 0·01 M substrate. Gas 95% N_2–5% CO_2. Temperature 40°. ×—×, xylose; ●—●, glucose; △—△, galacturonic acid; ▲—▲, glucuronic acid; ⊙—⊙, arabinose; ◆—◆, α-glucuronic acid-1-phosphate; ⊡—⊡, galactose, β-glucuronic acid-1-phosphate, and no substrate.

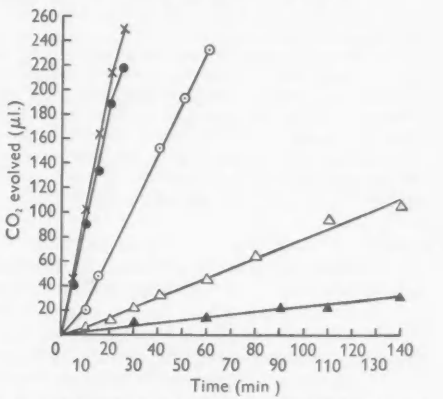

Fig. 2. Acid production from various substrates by cells previously grown on glucuronate. Flasks contained 60 μg. cell N in 2·25 ml. of bicarbonate saline. Side arms contained 0·5 ml. 0·01 M substrate. Gas 95% N–5% CO_2. Temperature 40°. ×—×, glucuronate; ●—●, galacturonate; ⊙—⊙, glucose; △—△, xylose; ▲—▲, arabinose, galactose, α- and β-glucuronic acid-1-phosphates, and no substrate.

to any extent while glucuronic and galacturonic acids and arabinose were used after a more or less short lag period. This was interpreted to mean that the cells formed adaptive systems for the fermentation of these substrates while in resting suspension.

In Fig. 2 is shown the results of shaking cells grown on glucuronic acid with the above substrates. Glucuronate and galacturonate were fermented immediately and at the same rate. Glucose was also fermented immediately, but at a somewhat slower rate, while xylose, arabinose, galactose and α- and β-glucuronic acid-1-phosphates were fermented either very slowly or not at all. The results of these

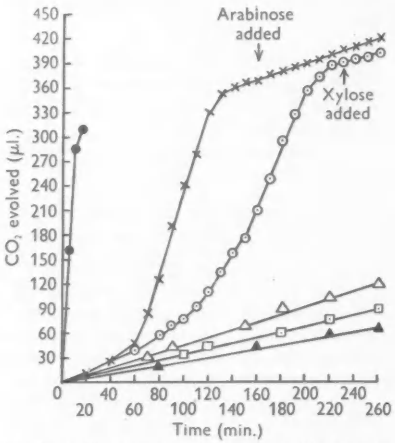

Fig. 3. The utilization of xylose and arabinose, by cells grown on glucose and adapted to glucuronic and galacturonic acids. Vessels with two side arms used. Each vessel contained 300 μg. cell N in 2·25 ml. bicarbonate saline. Side arms contained 0·5 ml. 0·01 M substrate or 0·5 ml. buffer. Gas 95% N_2–5% CO_2. ●—●, glucose; ×—×, galacturonic acid; ⊙—⊙, glucuronic acid; △—△, xylose; ⊡—⊡, arabinose; ▲—▲, no substrate.

experiments suggested that the uronic acids were not metabolized by a primary step involving decarboxylation to the homologous pentoses. However, since the preparation of the cells involved growth, these results may have been caused by the selection of certain cells during growth. Because of this, it was not possible to state definitely that the system for xylose fermentation was replaced by the system for glucuronate fermentation when cells were grown on glucuronate, or that the failure of cells grown on glucuronate to ferment xylose, showed that xylose was not an intermediate in glucuronate metabolism. It was possible, however, to test both these points under conditions in which growth could not be a major factor in the results. The first point was investigated as follows. It had been found that cells which had been grown on glucose were able to form adaptive systems to ferment both glucuronic and galacturonic acids, but did not ferment xylose, arabinose or galactose (Fig. 3). However, when the pentoses were added to cells which had developed adaptive systems to the corresponding uronic acids, no further increase in gas production was observed (Fig. 3), and from this it was concluded that the fermentation of the uronic acids did not proceed directly through the pentose. In order to investigate the second point, cells grown on xylose were adapted to glucuronate and then allowed to metabolize xylose (Fig. 4). From this experiment it was clear that the formation of a system for the metabolism of glucuronate did not in itself affect the utilization of xylose.

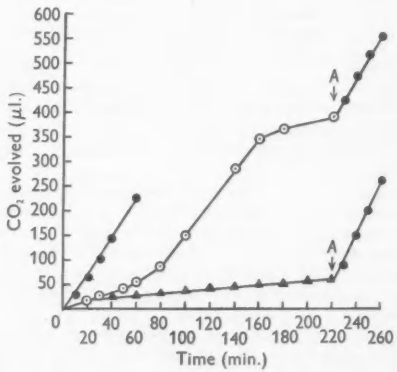

Fig. 4. The utilization of xylose by cells previously grown on xylose and adapted to glucuronate metabolism. Vessels contained 120 μg. cell N in 2·25 ml. bicarbonate saline. Two side arms contained the substrates 0·5 ml. of 0·01 M in each. Gas 95% N_2–5% CO_2. Temperature 40°. Xylose tipped into glucuronate and into no substrate control, at *A*. ●—●, xylose; ⊙—⊙, glucuronate; ▲—▲, no substrate.

The rate of formation, in resting cells, of the adaptive system for the fermentation of glucuronate

In many experiments it was found that there was considerable variation in the period before which cells, grown on xylose, were able to ferment glucuronate. This period was increased by using cells which had been well washed (Fig. 5). The original activity could be restored by adding to the well washed cells some of the first washings (Fig. 6), thereby showing that the decrease in activity shown in Fig. 5 was not owing to the cells suffering damage. Also the effect was not due to the presence of amino-acids since the addition of a mixture of amino-acids (casein hydrolysate + 3% tryptophan) in a quantity equal to the amount of nitrogen determined in the cell washings (8–15 μg./200 μg. cell nitrogen) had no effect on the rate of formation of the system for glucuronate.

The period could be reduced considerably by 'priming' the cells with small quantities of either glucose or xylose (Table 2), but it was also found

that such an effect was obtained only if glucuronate was present during the period of active metabolism of the added 'priming' substrate (Fig. 7). This was interpreted to mean that an energy source was necessary to enable glucuronate to be metabolized. Addition of ATP (100 μg./manometer) produced a similar shortening of the period (Table 3). This lag period could be abolished almost completely by growing cells overnight, first on glucuronate and then on either xylose or glucose. Such an experiment is shown in Fig. 8, and it is seen that the rate of fermentation of glucuronate ($q_{CO_2}^{N_2} = 400$) was equal to that of xylose ($q_{CO_2}^{N_2} = 404$) and was also similar to the rate for cells grown on glucuronate ($q_{CO_2}^{N_2} = 450$). When these cells were once again cultured on glucuronate the system for xylose was lost, and the metabolic pattern followed that shown in Fig. 2.

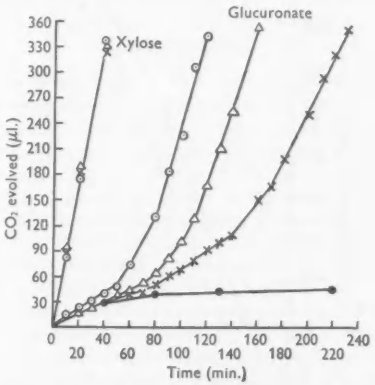

Fig. 5. The effect of extensive washing of cells previously grown on xylose, on the rate of adaptation to glucuronate metabolism. Vessels contained 135 μg. cell N in 2·3 ml. bicarbonate saline. Side arms contained 0·5 ml. 0·01 M substrate. Gas 95 % N_2–5 % CO_2. ⊙—⊙, not washed; △—△, twice washed; ×—×, washed six times; ●—●, controls, no substrate.

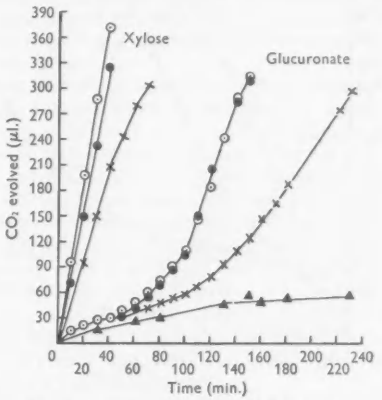

Fig. 6. The effect on the rate of adaptation to glucuronate metabolism of adding the first washings to well washed cells. Each vessel contained 100 μg. cell N in 2·25 ml. of bicarbonate saline. The side arms contained 0·5 ml. 0·01 M substrate. The washings, included in one of the duplicate sets of flasks, were added with the cell suspension. Gas 95 % N_2–5 % CO_2. ⊙—⊙, unwashed cells; ●—●, cells washed six times, +first washings; ×—×, cells washed six times; ▲—▲, no substrate.

With cells grown on glucuronate, however, the addition of glucose (0·1 ml. 0·01 M solution), or glucuronate (0·1 ml. 0·01 M solution) or ATP

Table 2. *Effect of adding a small quantity of xylose on the production of the adaptive system for glucuronate metabolism in cells grown on xylose*

(Each manometer contained a total volume of 0·6 ml. in the side arm and 200 μg. of cell nitrogen in the flask. The total volume of liquid was 2·85 ml. bicarbonate saline. The values represent μl. of gas evolved.)

	Carbohydrate in side arm of manometer flask			
Time (min.)	D-Xylose (0·5 ml. 0·01 M)	D-Xylose (0·1 ml. 0·01 M) +sodium D-glucuronate (0·5 ml. 0·01 M)	Sodium D-glucuronate (0·5 ml. 0·01 M)	D-Xylose (0·1 ml. 0·01 M)
0	0	0	0	0
10	140	75	16	65
20	274	101	17	71
30	351	113	22	69
40	356	127	25	68
50	—	148	30	69
60	—	180	38	71
70	—	225	51	72
80	—	291	60	74
90	—	352	73	76
100	—	396	85	78

(100 μg.), did not promote the fermentation of xylose. In each instance, after the added carbohydrate had been fermented, the rate of gas production from xylose resembled that shown in Fig. 2.

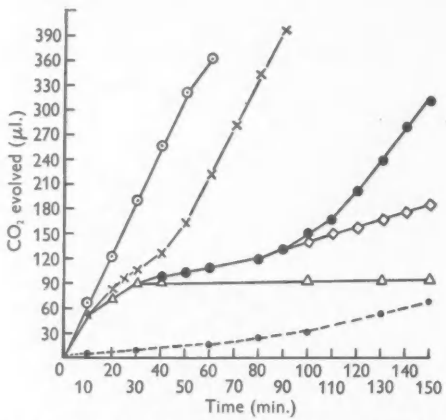

Fig. 7. The effect of the metabolism of xylose on the rate of formation of the adaptive system for glucuronic acid fermentation in cells grown on xylose. Vessels with two side arms used. Vessels contained 115 μg. cell N in 2·3 ml. bicarbonate saline in the centre compartment. Side arms contained 0·1 ml. 0·01 M-xylose or 0·1 ml. buffer; and 0·5 ml. 0·01 M-xylose, or 0·5 ml. 0·01 M-glucuronate, as required. Gas 95 % N_2–5 % CO_2. ⊙—⊙, xylose (0·5 ml. 0·01 M); ×—×, xylose (0·1 ml. 0·01 M), and sodium glucuronate (0·5 ml. 0·01 M) added at start; ●—●, xylose (0·1 ml. 0·01 M) added at start, glucuronate (0·5 ml. 0·01 M) added after 20 min.; ◇—◇, xylose (0·1 ml. 0·01 M) added at start, glucuronate (0·5 ml. 0·01 M) added after 30 min.; △—△, xylose (0·1 ml. 0·01 M); •- - - -•, glucuronate (0·5 ml. 0·01 M) added at start, no xylose present.

Experiments with other cultures

Cells of *Esch. coli* type 86, previously grown on xylose, developed an adaptive system for the fermentation of glucuronate under the conditions described above. The rate of formation of this system was increased by the presence of xylose, as in Fig. 7. When grown on glucuronate, the cells were unable to ferment xylose. When cultured again on

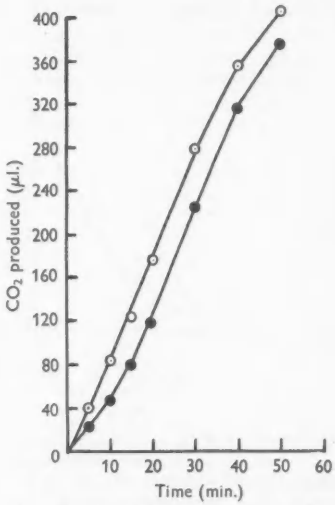

Fig. 8. The utilization of glucuronic acid and xylose by cells grown overnight firstly on glucuronic acid and then on xylose. Flasks contained 150 μg. cell N in 2·25 ml. of bicarbonate saline. Side arms contained 0·5 ml. of 0·01 M substrate. Gas 95 % N_2–5 % CO_2. Temperature 40°. ⊙—⊙, xylose; ●—●, glucuronate. $q_{CO_2}^{N_2}$ (xylose) = 404. $q_{CO_2}^{N_2}$ (glucuronic acid) = 400.

Table 3. *Effect of ATP on the utilization of glucuronate by washed suspensions of cells grown on xylose*

(Each manometer flask contained the substrate in the side arm and in the main compartment 120 μg. cell nitrogen in 2·3 ml. bicarbonate saline. Values in μl. gas evolved.)

	Contents of side arm			
Time (min.)	D-Xylose (0·5 ml. 0·01 M) + 0·1 ml. buffer	D-Glucuronate (0·5 ml. 0·01 M) + 0·1 ml. buffer	D-Glucuronate (0·5 ml. 0·01 M) + 100 μg. ATP in 0·1 ml.	0·5 ml. Buffer + 100 μg. ATP in 0·1 ml.
0	0	0	0	0
5	31	12	15	6
10	72	12	19	16
15	105	20	23	22
20	138	20	28	19
30	224	18	34	28
40	281	28	55	39
50	308	33	94	40
60	343	45	167	45
70	345	56	262	53
80	—	74	392	56
90	—	90	485	60

xylose, these cells did not ferment glucuronate immediately, but only after a lag period, as in Fig. 1. *Aerobacter aerogenes* 8197, when grown on xylose, did not ferment glucuronate and the addition of a trace of xylose, as a 'primer', was without effect. The culture would, however, grow in a medium containing glucuronate as the sole carbon source.

DISCUSSION

It was mentioned in the introduction that one hypothesis for the mechanism of the formation of pentoses involved an initial decarboxylation of the homomorphous uronic acid. The results presented above show that, as far as the coliform organisms tested are concerned, there is not such a simple relation between the metabolism of uronic acids and of the homologous pentoses. While such studies with intact cells involving simultaneous adaptation are necessarily subject to the limitations discussed fully by Stanier (1950), the experiments shown in Figs. 2 and 3 demonstrate that although the sugar acids can be metabolized the initial step does not involve a primary decarboxylation to the pentose. The lag in fermentation of the uronic acids by cells grown either on glucose or xylose is considered to be due to the formation of an adaptive system. This term is used in the sense discussed by Reiner & Spiegelman (1947), Spiegelman (1950) and Stanier (1951) and is taken to mean that certain essential units of the enzyme system(s) involved must be already present in the cells, and must increase during the metabolism of the substrates, in this instance the uronic acids.

This system, which is considered, at least in the initial stage, to be the same for both glucuronic and galacturonic acids, was formed in washed, resting cells, in the absence of fixed exogenous nitrogen, under anaerobic conditions, and was developed very rapidly. This appears to be the first instance of the demonstration of such a carbohydrase system in resting bacteria.

The fact that cells grown either on glucose or xylose were able to ferment glucuronate, implies that a small quantity of the necessary enzyme system is always present in the cells, and suggests that the metabolism of uronic acids may be of fundamental importance to these organisms. Xylose was fermented only to a small extent, if at all, by cells grown on glucuronate or glucose and since this low rate was not increased by supplying sources of energy in the form of glucuronate, glucose and ATP, it is considered that energy alone is not sufficient for the development of the xylose-fermenting system, which increases only during growth. The results in Fig. 4 show that the development of an adaptive system for the metabolism of glucuronic acid in resting cells grown on xylose does not involve a loss or decrease of the system for the metabolism of xylose. This does not entirely agree with the results of Spiegelman & Dunn (1947) who found that the formation of galactozymase in a strain of *Saccharomyces cerevisiae*, previously grown on glucose, caused a fall in glucozymase activity. However, Wainwright & Pollock (1949) found that the adaptive production of tetrathionase in resting cells of *Esch. coli* did not cause a reduction in the activity of the nitratase system already present.

The marked acceleration of formation of the system for glucuronate metabolism, brought about by xylose, glucose and ATP is similar to that reported by Spiegelman, Reiner & Cohnberg (1947) who showed that, in yeast grown on glucose, formation of galactozymase and maltozymase was facilitated by addition of small amounts of glucose. These results imply that only a source of energy was necessary, but since the presence of some factor which could be washed from the cells also enhanced the rate of adaptation, it does not appear that an energy source alone is sufficient. This conclusion remains uncertain, however, in the absence of further knowledge of the factor.

It is of interest to note that in Fig. 6 the rate of utilization of xylose by well washed cells is less than the rate for unwashed cells, or for supplemented washed cells. This result was not always observed (cf. Fig. 5) in this type of experiment, but it had been previously found that the rate of utilization of xylose by cells grown on xylose might vary considerably from the usual $q_{CO_2}^{N_2}$ (xylose) of 400–450. In one instance a value of $q_{CO_2}^{N_2}$ (xylose) of 250 was obtained. Perhaps the factor removed by washing is also involved in the metabolism of xylose.

So far, comparisons have been made with only two stock strains of coliform organisms and of these only *Esch. coli* N.C.T.C. no. 86 behaved in a similar manner to the strains investigated above. *Aerobacter aerogenes* N.C.T.C. 819 did not ferment glucuronic acid after growth on xylose. These results are too few to permit of any definite conclusions, but it would appear that all coliform organisms do not behave in the same way in metabolizing glucuronic acid.

SUMMARY

1. Washed suspensions of strains of *Escherichia coli* previously grown on xylose are able to form adaptive systems for the fermentation of glucuronic and galacturonic acids and L-arabinose. Glucose was fermented immediately, but galactose and α- and β-glucuronic acid-1-phosphates were not fermented.

2. Cells grown on glucose could ferment glucose, and formed adaptive systems for glucuronate and galacturonate, but did not ferment galactose,

xylose, arabinose or α-glucuronic acid-1-phosphate.

3. Cells grown on glucuronate could ferment glucose, glucuronate and galacturonate but not galactose, xylose, arabinose or α- and β-glucuronic acid-1-phosphate.

4. In the case of cells grown on xylose the adaptation period was greatly shortened by adding glucose, xylose or adenosinetriphosphate, and also by the presence of a 'factor' which could be washed from the cells.

5. Cells grown on glucuronate could not be made to ferment xylose by the addition of glucuronate, glucose or adenosinetriphosphate.

6. The possible significance of these results is discussed.

The author wishes to thank Dr A. E. Oxford for his helpful criticisms during the course of this work.

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Tracer Studies with the B_{12} Vitamins

1. NEUTRON IRRADIATION OF VITAMIN B_{12}

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The presence of cobalt and phosphorus in the vitamin B_{12} molecule invites attempts to label the vitamin with radioactive isotopes to facilitate metabolism studies. Soon after our isolation of crystalline vitamin B_{12} we tried direct chemical exchange with radioactive cobalt compounds and radioactive phosphate (Fantes, Page, Parker & Lester Smith, 1949). Not surprisingly, in the light of later knowledge about the constitution of vitamin B_{12}, no significant radioactivity was introduced into the molecule during these experiments. These observations have recently been confirmed and extended by Baldwin, Lowry & Harrington (1951), and also by Boos, Rosenblum & Woodbury (1951), who found no evidence of exchange with ^{60}Co after 1 or 3 months contact.

About the same time we considered direct irradiation of vitamin B_{12} in the atomic pile, bearing in mind the observation of Ball, Solomon & Cooper (1949) that the Szilard-Chalmers reaction does not go to completion, and their claim to have obtained ^{35}S-labelled cystine in small yield by direct neutron bombardment of cystine. We were advised that the experiment was not worth attempting with valuable material, but later Dr A. G. Maddock suggested that irradiation with thermal neutrons might be expected to induce some radioactivity in the vitamin B_{12} molecule without causing excessive decomposition of the material if it were properly prepared.

RESULTS

Neutron irradiation: first experiment

Anhydrous crystalline vitamin B_{12}, sealed *in vacuo* in a silica tube, was exposed to a low neutron flux in British Experimental Pile (BEPO) for 4 weeks. After irradiation the material was unchanged in appearance and measurement of the colour of its

aqueous solution indicated little destruction; it was purified by solvent extraction and two crystallizations from aqueous acetone. The recrystallized product was still microbiologically active.

At this stage additional vitamin B_{12} was added as 'carrier'. The specific activity fell rapidly with successive recrystallizations. It then occurred to us that the cyano group attached to the cobalt atom might have been split off by the neutron bombardment, as it is by visible light. If this were so, the radioactive species would be vitamin B_{12b}, which might be expected to separate partially from the 'carrier' vitamin B_{12} on crystallization. To test this hypothesis a trace of potassium cyanide was added and recrystallization was continued. The specific activity measured after each second recrystallization was essentially constant at 0·006 μc./mg. Any radioactivity that might have been induced in the phosphorus atom would have decayed to a negligible amount during the 4 months since the irradiation.

The mother liquors accumulated before the addition of cyanide were also treated with a trace of potassium cyanide. After four recrystallizations the specific activity was 0·016 μc./mg., not significantly changed by the four additional recrystallizations. It will be noted that the specific activity of this material from the mother liquors was higher than that from the former series of crystallizations, which again suggests that radioactive vitamin B_{12b} had accumulated in the mother liquors before its reconversion into vitamin B_{12} by cyanide.

Chromatography on paper

Material from each series of crystallizations was chromatographed on paper, with *sec.*-butanol as solvent. Exploration of the paper strip with a monitor Geiger-Müller tube showed significant radioactivity only at the red vitamin B_{12} spot. The specific activity of material recovered from the paper strip was the same, within the limits of experimental error, as that of the samples taken for chromatography.

Conversion to vitamin B_{12c}

In order to remove any final suspicion that radioactivity may reside in some persistent impurity, it is customary to demonstrate unaltered specific activity on converting the compound in question into a derivative that can be purified in a different way. Accordingly, the remaining 12-times recrystallized vitamin B_{12} was converted via the sulphite adduct of vitamin B_{12b} into vitamin B_{12c}. Since very little of the sulphite adduct is extracted by benzyl alcohol at pH 8·5, opportunity was taken to extract possible basic impurities by this means. After treatment with nitrite as described in the Experimental section, the resulting vitamin B_{12c} was itself extracted with benzyl alcohol from the solution at pH 8·5, thus tending to eliminate possible acidic impurities. The product was precipitated with ether and crystallized from aqueous acetone. Allowing for radioactive decay during the 10 months since the starting material was assayed, the first crop appeared to have a slightly diminished specific activity, but that of the second crop was unchanged.

Neutron irradiation: second experiment

The neutron irradiation was repeated with a somewhat larger sample of vitamin B_{12}, so as to provide enough material for successive recrystallizations without addition of 'carrier'. It was also hoped to carry out the purification speedily enough to detect possible activation of the phosphorus as well as the cobalt atom. On this occasion cyanide was added at the beginning, yet despite the absence of dilution by 'carrier' the specific activity fell further than in the previous experiment. Counts were taken (see Experimental) with and without the interposition of a piece of lead foil to intercept most of the soft β-radiation from ^{60}Co, while allowing the passage of much of the hard radiation from ^{32}P. The apparatus was calibrated with sources of ^{60}Co and ^{32}P so that the activities due to each isotope could be calculated. The total activity induced in the 20 mg. of vitamin B_{12} was 3·9 μc. due to ^{60}Co and 1·6 μc. due to ^{32}P. These are very close to the expected values. Both radioactive isotopes were still present in the recrystallized vitamin B_{12}. The specific activities became fairly constant, at 0·012 μc./mg. due to ^{60}Co and 0·005 μc./mg. due to ^{32}P, from the fourth to the eighth crystallization, although on subsequent fractionation about half this activity was eliminated. The persistent contamination probably arose from omission to purify this batch by an initial extraction with phenol; certainly, washing a phenol extract of the eight times crystallized material with acid and alkali diminished the ^{60}Co activity to 0·008 μc./mg.

Partition chromatography on kieselguhr columns and on filter paper

To obtain more material, the accumulated mother liquors were purified by solvent extraction, followed by six crystallizations, to a ^{60}Co activity of 0·009 μc./mg. By this time the ^{32}P activity had been lost by decay. The product was chromatographed on damp kieselguhr, with wet *n*-butanol as mobile phase as described by Lester Smith *et al.* (1952). The product from the red band had a specific activity of 0·0065 μc./mg., and this was unchanged on repeating the partition chromatography.

Approximately 0·5 mg. of this product was chromatographed again, on paper. A strong solution was applied as a streak about 6 cm. long

across a sheet of filter paper, which was developed with water-saturated *sec.*-butanol in the presence of a trace of hydrogen cyanide in the tank. The fairly compact single developed zone was cut into head, middle and tail portions, which were separately eluted with water. The eluates were dried directly on planchettes for counting. Afterwards the vitamin B_{12} was washed off for spectrophotometric assay. The specific activities of all three fractions were 0·0075 μc./mg. In view of the small amounts available for assay, this value cannot be considered significantly different from that of the starting material.

DISCUSSION

The evidence is fairly conclusive that direct activation of vitamin B_{12} was effected by bombardment with thermal neutrons in both experiments. The activity associated with the vitamin B_{12} was only a small fraction of the total activity induced in the sample, and the final specific activities were unfortunately too low for the products to be of value in biological experiments. The lower specific activities in the second experiment may have resulted from accidental omission to seal the sample *in vacuo*, or alternatively from the use of microcrystalline material, for in the first experiment we had used vitamin B_{12} crystals about 1 mm. in length.

After this paper had been written the communication by Anderson & Delabarre (1951) appeared. They irradiated vitamin B_{12} in the more powerful neutron flux of the Chalk River Pile and purified the product by chromatography. They make the surprising claim that about 80 % of the induced radioactivity was associated with vitamin B_{12}.

EXPERIMENTAL

Anhydrous crystalline vitamin B_{12} (2·5 mg.) sealed *in vacuo* in a quartz tube was exposed in BEPO for 4 weeks to a flux of approximately $0{\cdot}5 \times 10^{11}$ neutrons/sq.cm./sec. The irradiated material was dissolved in 25 ml. of 0·04 N-HCl and the solution was shaken with phenol (5 g.). The phenol extract containing nearly all the colour was washed with 0·04 N-HCl (5 ml.). Ether was then added (15 ml.), and the mixture was shaken with several small portions of water (total about 10 ml.) till all the colour was removed. The solution was concentrated to about 0·25 ml. under reduced pressure, then acetone was added to faint turbidity to induce crystallization. It was recrystallized in the same manner. The product then contained 2·0 mg. of anhydrous vitamin B_{12} (assessed colorimetrically). It was mixed with an additional 4·9 mg. of vitamin B_{12}. This and subsequent preparations were recrystallized twice from aqueous acetone, then twice from water, twice from aqueous acetone and so on. Crystallization from water was effected by dissolving in one or two drops of hot water and allowing to stand overnight; if necessary some water was allowed to evaporate. The small volume of mother liquor was removed by placing a narrow strip of filter paper in the tube with its pointed end touching the mixture of crystals and solution.

Radiometric measurements were made by dissolving part or all of the sample in 10 ml. of water for counting in the jacketed Geiger-Müller tube described by Veall (1948); vitamin B_{12} was estimated colorimetrically on the same solution. Radioactive cobalt (1 μc.) in 10 ml. gave 12 400 counts/min.; the various vitamin B_{12} solutions gave around 100 counts/min.

Conversion to vitamin B_{12c}

The conversion to vitamin B_{12c} was carried out as follows: 1·5 mg. of the neutron-irradiated vitamin B_{12} and 30 mg. potassium metabisulphite ($K_2S_2O_5$) were dissolved in 3 ml. of water. The solution was adjusted with HCl to pH 3·5, approximately, and after it had stood 1·25 hr. at room temperature it was brought to pH 8·5 with alkali. Two extractions, each with 3 ml. of benzyl alcohol, were carried out; the phases were separated by Pasteur pipette after centrifuging. The aqueous residue was acidified to about pH 3 and concentrated slightly under reduced pressure. Several small additions of acid and water were made and distillation was repeated until all SO_2 was removed and the solution remained acid. $NaNO_2$ (10 mg.) was then added, the pH was adjusted to about 4 and the solution was warmed gently for 5 min. Alkali was again added and the vitamin B_{12c} was extracted from the 2 ml. of aqueous solution at pH 8·5 with two 3 ml. portions of benzyl alcohol. Ether (12 ml.) and a few drops of water were shaken vigorously with the bulked extracts; on centrifuging, all the colour appeared in the few drops of aqueous layer, to which, after separation, acetone was added to faint opalescence to induce crystallization.

Differential counting of ^{32}P *and* ^{60}Co

In the second experiment differential counting for ^{60}Co and ^{32}P was carried out with a thin end-window Geiger-Müller tube. The planchette was placed in a position farther away from the window than usual to allow room for the interposition of a piece of lead foil (thickness 0·12 mm.) as absorber. A cobalt standard gave 72 600 counts/min. without and 2900 counts/min. with the absorber. A phosphorus standard gave 133 000 counts/min. without and 28 800 counts/min. with the absorber. The vitamin B_{12} after successive crystallizations gave about 200 counts/min. without and 20 counts/min. with the absorber.

Chromatography on paper

The vitamin B_{12} was dissolved in a drop or two of water, and applied as a spot or streak to a strip or sheet of Whatman no. 4 filter paper. To prevent undue spreading the solution was applied in several portions to the same area, air-drying the paper in between. Development by the usual downward method was effected with water-saturated *sec.*-butanol. The R_F value was about 0·2 but the solvent was allowed to run off the bottom of the paper.

SUMMARY

1. Vitamin B_{12} has been activated by bombardment with thermal neutrons in the atomic pile.

2. In each of two experiments a small proportion of the induced activity remained associated with the vitamin B_{12} after thorough purification by solvent extraction, recrystallization and chromatography.

3. The second experiment provided evidence that the phosphorus as well as the cobalt in the vitamin B_{12} had become radioactive.

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Tracer Studies with the B_{12} Vitamins

2. BIOSYNTHESIS OF VITAMIN B_{12} LABELLED WITH ^{60}Co AND ^{32}P

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In 1949 we attempted to obtain radioactive vitamin B_{12} from the livers of rats and pigs receiving ^{60}Co salts in their diets (Braude, Free, Page & Lester Smith, 1949; Cuthbertson, Free & Thornton, 1950). These attempts failed because the livers contained negligible amounts of vitamin B_{12}. We recognized at the time that it would have been preferable to use ruminants, but such animals were not available to us. Subsequent work suggests that we ought to have extracted the faeces rather than the livers of our animals.

About the same time we succeeded in preparing very small amounts of radioactive vitamin B_{12} concentrates by fermentations with *Aerobacter aerogenes* isolated from hen faeces. More recently we have returned to this problem, using *Streptomyces griseus*; in the meantime the preparation by this method of [^{60}Co] vitamin B_{12} with low or moderately high specific activity has been reported (Chaiet, Rosenblum & Woodbury, 1950; Rosenblum & Woodbury, 1951).

RESULTS

Preparation of vitamin B_{12} containing ^{60}Co

To avoid diminishing the specific activity of the added radioactive cobalt, we used a semi-synthetic medium (Dulaney, 1948) shown to be nearly free from cobalt. We added only about 0·2 p.p.m. of radio-cobalt, thus minimizing any radiation hazard and securing efficient conversion of cobalt to vitamin B_{12}. The fermentation liquors were worked up to yield a few mg. of crystalline vitamin B_{12} by a simple procedure described in the Experimental section. The latest fermentation with ^{60}Co at 3·5 mc./mg. has yielded vitamin B_{12} with a specific activity of 120 μc./mg., which is considerably higher than any previously recorded (Rosenblum & Woodbury, 1951).

Preparation of vitamin B_{12} containing ^{32}P

It was of interest to attempt the preparation of vitamin B_{12} labelled with radioactive phosphorus, since this would be safer for projected human metabolism experiments than vitamin B_{12} containing radioactive cobalt with its much longer half-life and penetrating gamma radiation. It was foreseen that it would be difficult to prepare material with adequate specific activity because preliminary experiments had shown that *Streptomyces griseus* needs a moderately high level of phosphate in the medium, growth and vitamin B_{12} production being impaired when the total phosphorus level is reduced to about 0·01 %.

It was decided to use 10 mc. of ^{32}P and to aim at preparing about 1 mg. of the radioactive vitamin. It was then almost immaterial what volume of medium was used for the fermentation; the smaller the volume, the higher the specific activity of the resulting vitamin B_{12} would be, but the greater the proportion of ordinary vitamin B_{12} that would have to be added.

The radioactive phosphate was added aseptically 24 hr. after fermentation started. This was done in the hope that part of the phosphorus in the medium would already have been taken up by the mould and converted into organic phosphorus compounds that would not exchange with the added ^{32}P. This hope was not realized; the specific activity of the product was the same as that from a pilot run with

the ^{32}P added before inoculation. The yield of crystalline vitamin B_{12} was 0·91 mg. with a specific activity of 0·35 μc./mg. Less than one part in 10 000 of the ^{32}P added was present in the vitamin B_{12} produced by this fermentation.

EXPERIMENTAL

Fermentations with ^{60}Co

Several fermentations have been carried out with ^{60}Co of the highest specific activity available at the time, namely:

Specific activity of ^{60}Co (mc./mg.)	Specific activity of resulting vitamin B_{12} (μc./mg.)
0·35	14
0·50	18·5
3·5	120

A synthetic medium (Dulaney, 1948) containing 0·2 p.p.m. of ^{60}Co was employed. The fermentations were carried out in bottles containing 3–15 l. of medium.

After autoclaving and inoculating with a vegetative culture of *S. griseus*, the bottles were fixed in a water bath thermostatically controlled at 27° and the contents were stirred and aerated for 4 days. The broths usually contained 0·5–1 μg. of vitamin B_{12}/ml. assessed by microbiological assay with an *Escherichia coli* mutant (Harrison, Lees & Wood, 1951).

Extraction of vitamin B_{12} *from fermentation liquors*

The broths were processed in slightly different ways; the following procedure is the simplest. KCN (1 g.) was added to 2·5 l. of the harvested broth. The mixture was allowed to stand 1 hr. at pH 8 and then (for convenience) overnight at pH 5. It was then boiled for 5 min. and filtered with the aid of kieselguhr; the residue was washed with a little water.

Charcoal (30 g., Sutcliffe Speakman no. 5 as purchased) and 15 g. of kieselguhr were slurried with water and made into a bed on a 14 cm. Büchner funnel. One-third of the broth plus washings was run slowly through this bed, followed by 400 ml. of 6% phenol to elute impurities and permit adsorption of additional vitamin B_{12}. Two further similar portions of broth and 6% phenol were run through the same bed. The adsorbate was eluted *in situ* with 1·5 l. of 60% (v/v) aqueous acetone. This eluting agent gives a clean product that can be worked up with little additional loss, although it does not remove the whole of the activity from the charcoal. Continued elution with 300 ml. of 90% phenol brought off additional vitamin B_{12} equivalent to about one-quarter of that originally present in the broth.

The acetone eluate was concentrated under reduced pressure to 32 ml. after addition of a trace of cyanide. The concentrate was shaken with 40 ml. of butanol and 16 g. of $(NH_4)_2SO_4$. Some black sludge was precipitated; it caused an emulsion, which was broken by filtering the whole mixture through kieselguhr. The sludge was redissolved in a little dilute $NaHCO_3$ solution and added to the aqueous layer, which was given two further extractions each with 50 ml. of butanol. The combined butanol extracts were shaken with successive small portions of water acidified to about pH 3 with HCl. Washing was continued until no further pink colour was removed.

The combined aqueous extracts were concentrated to 5 ml. and extracted twice with 1 vol. of phenol-benzene 1:2. The solvent extracts were washed with dilute $NaHCO_3$ and with water. The bulked solvent extracts were mixed with about 2 ml. of acetone and 40 ml. of ether. After 1 hr. the red precipitate was collected by centrifuging. It was redissolved in about 1 ml. of ethanol, centrifuged from a trace of insoluble matter and reprecipitated with ether. The precipitate was dissolved in a few drops of water and acetone was added; crystallization occurred almost immediately. The product, after recrystallization from aqueous acetone, was characterized as vitamin B_{12} by its absorption spectrum. Yields of crystalline vitamin B_{12} equivalent to 30–40% of that present in the broth have been obtained by this extraction procedure or similar ones.

Vitamin B_{12} *containing* ^{32}P

The medium (200 ml.) containing approximately 0·01% total P was distributed into five 250 ml. conical flasks. After autoclaving and inoculation with a vegetative culture of *Streptomyces griseus* the flasks were incubated at 27° on a rotatory shaker. At 24 hr. 2 mc. of ^{32}P in the form of sterile 'carrier-free' phosphate was added aseptically to each flask. Growth of mycelium was poor; subsequent experiments with lower levels of ^{32}P showed that this was not due to radiation damage but to the sub-optimal level of total P in the medium.

The broth was harvested at 4 days. It was adjusted to pH 4·5, heated in a boiling-water bath for 20 min., then filtered. The vitamin B_{12} titre was 0·5 μg./ml. KCN (0·1 g.) was added and 'carrier' vitamin B_{12} (1·8 mg. anhydrous). In view of this fortification, the charcoal step was omitted and the liquor was extracted directly with butanol in presence of $(NH_4)_2SO_4$ followed by the other step described above.

SUMMARY

1. ^{60}Co has been incorporated in vitamin B_{12} by fermentation with *Streptomyces griseus*.

2. The crystalline product had the very high specific activity of 120 μc./mg.

3. ^{32}P has been similarly incorporated in vitamin B_{12}.

4. Competing metabolic requirements for phosphorus resulted in a low efficiency of conversion and a specific activity of only 0·35 μc./mg.

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B_{12} Vitamins (Cobalamins)

1. VITAMINS B_{12c} AND B_{12d}

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During 1949 we isolated three red crystalline anti-pernicious anaemia factors from *Streptomyces griseus* fermentation liquors. The first of these was vitamin B_{12} itself, originally isolated by Rickes, Brink, Koniuszy, Wood & Folkers (1948) and a little later in these laboratories (Lester Smith & Parker, 1948). The next was vitamin B_{12b}, the second factor we obtained from liver; it was later crystallized by Pierce, Page, Stokstad & Jukes (1949). The third was vitamin B_{12c}, which has been referred to under its temporary designation 'vitamin B_{12x}' at scientific meetings (Lester Smith, Ungley, Mollin & Dacie, 1950; Buchanan, Johnson, Mills & Todd, 1950*a*, *b*; Anslow *et al.* 1950; Lester Smith *et al.* 1951). These were all obtained by methods of isolation unlikely to alter the factors chemically. In addition, however, we have obtained a number of other closely related factors by mild chemical treatments of these B_{12} vitamins (Lester Smith, Ball & Ireland, 1952).

On hydrolysis with 5N-HCl followed by shaking with *n*-octanol, vitamins B_{12} and B_{12c} gave red esters with identical absorption spectra, indicating that a major portion of the molecule is common to both (Fantes & Ireland, 1950).

Nature of vitamin B_{12c}

The yield of the new factor, vitamin B_{12c}, was very small (e.g. 3 mg. from 2500 l. of fermentation liquor), but it was enhanced (e.g. to 60 mg. from 2500 l.) at the expense of the vitamin B_{12b} by a chemical step introduced to simplify the purification procedures. This was treatment with nitrous acid, which, by deaminating amino-acids and peptides, facilitates their removal from the crude concentrate. Nitrous acid appears to have no effect on vitamin B_{12} itself, but it readily converts vitamin B_{12b} into vitamin B_{12c}. Since the former is basic, while vitamin B_{12c} (like vitamin B_{12} itself) is not, the reaction was at first interpreted as a straightforward deamination. This explanation became dubious when we found that the product, vitamin B_{12c}, could readily be converted into a basic factor, which we called vitamin B_{12d} (Lester Smith *et al.* 1951); it arose not only on catalytic hydrogenation but also from some mild hydrolytic procedures. Experiments along these lines, however, showed some puzzling features. Thus a solution of vitamin B_{12c} at pH 4·5 was not appreciably altered on autoclaving in a sealed ampoule, yet the same solution on autoclaving in a flask plugged with cotton wool showed partial conversion to the basic factor; the two could be separated by partition chromatography on kieselguhr or paper, and were identified by differences in their absorption spectra, partition coefficients and chromatographic behaviour (see below, p. 393). Again, merely concentrating a similar solution under reduced pressure at a much lower temperature caused complete conversion to the basic factor. Standing in the cold in alkaline solution caused little change, yet ionophoresis in agar jelly at pH 9 (Gordon, Keil & Šebesta, 1949) brought about complete conversion. Evidently an equilibrium was set up in acid or alkaline solutions, and complete conversion depended upon the removal of one component from the system; moreover, this component was clearly volatile to some degree. Accordingly, acidified solutions of vitamin B_{12c} were distilled into very dilute alkali and the distillates were tested. They gave a blue colour with acidified starch iodide solution, and more specific colour reactions for nitrite with *m*-phenylenediamine or with sulphanilic acid and α-naphthylamine. A roughly quantitative colorimetric assay with these latter reagents indicated 0·65 mole of nitrite per molecule. Part of the nitrous acid would have been lost by oxidation during the distillation. We therefore concluded that the molecule of vitamin B_{12c} incorporates one nitrite group, probably linked directly to the cobalt atom.

Further confirmation of this relationship is given by the observation that rapid conversion of vitamin B_{12c} to the basic factor occurs in the cold on treating acidified solutions with either sulphamic acid or urea, reagents that rapidly destroy nitrous acid. After 5 min., the reaction went to completion with sulphamic acid, but only about half-way with urea.

A compound, presumably identical with vitamin B_{12c}, has recently been described briefly by Kaczka, Wolf, Kuehl & Folkers (1951) under the name nitrocobalamin. The nomenclature of these compounds is discussed by Lester Smith, Ball & Ireland (1952).

It is somewhat surprising to find such a compound as vitamin B_{12c} arising from a fermentation (for, as mentioned above, it is present in concentrates that have not been treated with nitrous acid). However, it is not necessarily synthesized directly by the mould; a trace of nitrite in the fermentation liquor (possibly arising from oxides of nitrogen in the air flow) could convert part of the vitamin B_{12b} present into vitamin B_{12c}.

Nature of 'vitamin B_{12d}'

The work described above was done before anything was known of the constitution of vitamin B_{12}, and we believed that nitrous acid caused some chemical change in vitamin B_{12b} besides being added on reversibly. In that event, the denitrosation product, which we called vitamin B_{12d}, would differ from vitamin B_{12b}. The absorption spectra were identical, including the shift at alkaline pH values already recorded for vitamin B_{12b} (Brockman, Pierce, Stokstad, Broquist & Jukes, 1950). Such spectroscopic evidence would normally be accepted as evidence of identity; in this range of molecular dimensions, however, we considered that a small difference in structure would not necessarily alter the absorption spectrum appreciably. The need for such caution has since been emphasized by the observation of Buhs, Newstead & Trenner (1951) that the absorption spectrum of vitamin B_{12b} is not altered on conversion to thiocyanatocobalamin. Partition chromatography would probably be the most sensitive technique to reveal such small differences, and it did appear to separate vitamins B_{12b} and B_{12d}. Thus kieselguhr partition chromatograms of concentrates (run with wet *n*-butanol, see Experimental section) sometimes showed an additional slow band, i.e. four bands in all, due respectively to vitamins B_{12c}, B_{12}, B_{12b} and (we presumed) B_{12d}. The non-identity of vitamins B_{12b} and B_{12d} was apparently confirmed by partition chromatography of the crystalline preparations (1–2 mg.) on 1 cm. diameter columns of kieselguhr carrying half its weight of pH 4·5 buffer (2% potassium dihydrogen phosphate) with wet *n*-butanol as mobile phase. Vitamin B_{12b} gave a slow-moving band ($R = 0{\cdot}1$ approx.) with only a trace of a slightly faster one: 'vitamin B_{12d}' gave a band that appeared to correspond with the latter ($R = 0{\cdot}2$ approx.), accompanied by a trace of a slower band, while a mixture gave two nearly equal bands. It now seems that these results were fortuitous. A recent reinvestigation (Lester Smith, 1952*a*) has shown that chromatograms of these three patterns, as well as columns showing a single band, can all be produced at will from the same specimen of vitamin B_{12b} by making slight changes in the operating procedure.

When the mobile phase was altered to 15% phenol in *n*-butanol (saturated with water) to secure a higher *R* value (0·25), a single band was seen consistently. Under these conditions mixtures of vitamins B_{12b} and 'B_{12d}' could not be separated. Their identity was confirmed by tracer techniques, using 'vitamin B_{12d}' mixed with vitamin B_{12b} containing radioactive cobalt (Lester Smith, Hockenhull & Quilter, 1952). On butanol columns two bands appeared; both, however, were radioactive, and the specific activities of the materials extracted from the two bands were the same. Thus the apparent separation of two entities was spurious. On the phenol-butanol column, however, only one band appeared: it was dissected into upper, middle and lower segments, which were separately eluted, yielding products of identical specific activities (within experimental error). This is strong evidence of identity, since a radioactive and a non-radioactive substance having partition coefficients differing by only a few per cent (i.e. not enough to cause separation into two bands) would still show a difference in specific activity between the top and bottom of the band (Udenfriend, 1950).

A similar experiment was carried out with a mixture of vitamin B_{12b} prepared from a fermentation with *Streptomyces aureofaciens* (kindly supplied by Dr T. H. Jukes of Lederle Laboratories) and radioactive 'vitamin B_{12d}'. The partition chromatogram showed a single red band ($R = 0{\cdot}25$); the upper and lower sections of this band gave materials of identical specific activity. Thus the two tracer experiments confirm the identity of 'vitamin B_{12d}' with vitamin B_{12b}, whether of natural origin or made from vitamin B_{12} by photolysis.

Basicity of vitamin B_{12b}

The basicity of vitamin B_{12b} was first revealed by its more complete extraction from alkaline than from neutral or acidic solutions by organic solvents, such as benzyl alcohol or phenol-butanol mixtures (Lester Smith, Ball & Ireland, 1952). Samples crystallized from neutral solutions behaved like salts of bases on electrometric titration in aqueous solution. The free base was prepared by passing an aqueous solution through a column of strongly basic anion-exchange resin (Experimental resin FF/X4 supplied by Permutit Ltd.), followed by crystallization from aqueous acetone. An aqueous solution (0·2%) of vitamin B_{12b} so treated had a pH value around 9 and consumed one equivalent of acid on electrometric titration (Fig. 1); the pK_a value was 7·5 (see also Kaczka, Wolf, Keuhl & Folkers, 1950).

Absorption spectra

The infrared absorption spectra of vitamins B_{12c} and B_{12b} are given in Fig. 2; the measurements were made by Dr N. Sheppard at the University of Cambridge. The main ultraviolet absorption band of vitamin B_{12c} in neutral aqueous solution occurs at

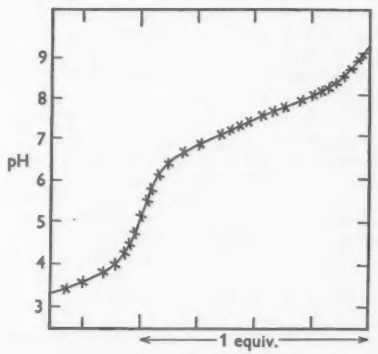

Fig. 1. Electrometric titration of vitamin B_{12b} (2·14 mg. vitamin B_{12b} base in 1·0 ml.).

352 mμ., that of vitamin B_{12b} at 351 mμ.; the former is also distinguishable by its greater breadth, the half-band width being about 42 mμ. compared with 30 mμ. for vitamin B_{12b}. Since this behaviour could be exhibited by a mixture of factors with slightly different absorption maxima, vitamin B_{12c} was subjected to rigorous purification. Recrystallization ten times from aqueous acetone did not change its absorption spectrum, and partition chromatography on kieselguhr with *n*-butanol revealed no sign of heterogeneity.

A number of physical measurements have been undertaken on vitamins B_{12b} and B_{12c}. The measurements include polarography, optical rotation, electrometric titration and electrical conductivity, and they were carried out on a micro scale in the same way as those reported for vitamin B_{12} (Fantes, Page, Parker & Lester Smith, 1949).

Polarography of vitamins B_{12b} and B_{12c}

The polarographic behaviour of vitamins B_{12b} and B_{12c} (recrystallized ten times) are summarized in Tables 1 and 2.

In neutral solution, both vitamins B_{12b} and B_{12c} formed characteristic waves with pronounced maxima; the half-wave potential was approximately $-1{\cdot}50$ V. The maximum was only partly suppressed by the addition of 0·01 % of gelatin. In alkaline and acid solution, the wave was much lower and the maximum disappeared. This behaviour is similar to that observed for vitamin B_{12} itself and is associated with catalytic waves rather than with normal reduction steps. It provides additional evidence that the cobalt atom is bound in a complex and is not ionizable. This work was completed before the appearance of papers on the polarographic behaviour of vitamin B_{12} by Diehl, Sealock & Morrison (1950) and Diehl, Morrison & Sealock (1951). These authors, by working with a higher concentration of vitamin B_{12} (9×10^{-4} M as opposed to $0{\cdot}6 \times 10^{-4}$ M), were able to identify a reduction step at $-1{\cdot}12$ V in addition to the catalytic wave.

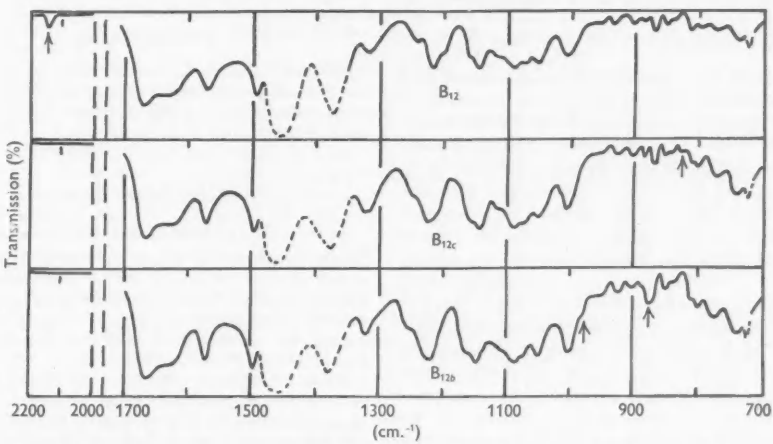

Fig. 2. Infrared absorption spectra of vitamins B_{12}, B_{12b} and B_{12c}. - - - - -, Nujol absorptions.

Table 1. *Polarographic behaviour of vitamin* B_{12b}

(The dropping mercury electrode at a pressure of 55·5 cm. of mercury and on open circuit in 0·1 N-KCl at 25° had a drop time of 3·13 sec.; the weight of the mercury dropping was 1·82 mg./sec. The half-wave potentials were measured against a saturated calomel electrode.)

Concn. of anhydrous vitamin B_{12b} (%)	pH value of solution	Half-wave potential (V.)	Diffusion current (μa.)	
			Maximum	Minimum
0·0060	7·4	−1·50	9·6	1·3
0·0058	10·8	−1·55	—	0·38

Table 2. *Polarographic behaviour of vitamin* B_{12c}

(Conditions as in Table 1.)

Concn. of anhydrous vitamin B_{12c} (%)	pH value of solution	Gelatin (%)	Half-wave potential (V.)	Diffusion current (μa.)	
				Maximum	Minimum
0·0093	3·3	0	−0·78	—	0·38
0·0090	3·3	0·01	−0·93	—	0·28
0·0096	7·2	0	−1·50	17·3	4·0
0·0090	7·2	0·01	−1·52	3·4	3·2
0·0090	9·6	0	−1·55	0·64	0·47
0·0085	9·6	0·01	−1·55	0·35	0·31

Electrical conductivity of vitamin B_{12c}

At a concentration of about $5{\cdot}9 \times 10^{-4}$ M, the specific conductivity of vitamin B_{12c} at 25° was 35 gemmhos. This value corresponds to an equivalent conductivity of about 59; the latter compares with a figure of 53 obtained for vitamin B_{12}. Vitamin B_{12c} is, therefore, weakly ionized in aqueous solution.

Magnetic susceptibility

A sample of vitamin B_{12c} was kindly examined by Dr Corona Trew, who found it to be diamagnetic, like vitamins B_{12} and B_{12b} (Diehl, Haar & Sealock, 1950; Wallmann, Cunningham & Calvin, 1951).

Optical rotation of vitamins B_{12b} *and* B_{12c}

The optical rotations have been measured in a 0·5 dm. micro-polarimeter tube having a capacity of 0·4 ml. The polarimeter was fitted with a direct-vision spectroscopic attachment, and an experimental compact source type cadmium lamp (kindly lent by Mr B. S. Cooper of the Research Laboratories of the General Electric Co.) was used as light source. The specific rotations for light of wavelength 643·8 mμ. of solutions at 20° containing about 0·5% of vitamin B_{12} (for comparison), vitamins B_{12b} and B_{12c} are listed in Table 3. It is difficult to obtain accurate results on these solutions, especially in a micro-tube, owing to their intense colour, which also prohibits the use of higher concentrations. Therefore a considerable number of individual readings were made on each and the means with their fiducial limits (at $P = 0{\cdot}05$) were calculated.

We were surprised to find that vitamin B_{12c} rotates in the opposite direction from vitamins B_{12} and B_{12b}. Brink *et al.* (1949) reported that vitamin B_{12} has a specific rotation of $-59° \pm 9°$ at 23° for light of wavelength 656·3 mμ.

Table 3. *Specific rotation of vitamins* B_{12}, B_{12b} *and* B_{12c} *in 0·5% aqueous solutions*

Vitamin	Mean specific rotations at 643·8 mμ. (°)	Fiducial range ($P = 0{\cdot}05$) (°)
B_{12}	−110	−134 to −87
B_{12b}	−19·5	−28·0 to −11·0
B_{12c}	+50	+41·5 to +59·0

Clinical activity

Vitamins B_{12c} and B_{12b} are just as effective as vitamin B_{12} itself against pernicious anaemia (Lester Smith *et al.* 1950; Ungley, 1951; Chalmers, 1951; Reid, 1951).

EXPERIMENTAL

Isolation of vitamin B_{12c}

The submerged fermentations of *Streptomyces griseus* were carried out on soybean medium (Kirkpatrick, 1947; Dulaney, Ruger & Hlavac, 1948). The fermentation liquor usually contained 0·5–1 μg./ml. of vitamin B_{12} activity assessed by microbiological assay with *Lactobacillus leichmannii* (Emery, Lees & Tootill, 1951). The broth was acidified with H_3PO_4 to pH 2·0–2·5 and filtered with the aid of kieselguhr (Hyflo Super-Cel) on a rotary filter. It was similarly refiltered after adjustment with NaOH to pH 7·5. The filtrate was heated at 100° for 15 min. to release B_{12} vitamins from bound forms.

The following purification procedure is typical; sometimes steps were omitted or modified slightly, or their order was changed. A portion of the liquor (4500 l.) was stirred for 0·5 hr. with charcoal (45 kg., Sutcliffe Speakman no. 5 as purchased). The charcoal was removed by means of a rotary filter precoated with kieselguhr and was washed with 6% aqueous phenol (2250 l.) to remove some adsorbed impurities. This washed adsorbate was then stirred with a further 4500 l. of fermentation liquor and was again washed with 6% phenol. These procedures were repeated with a third portion of liquor and of 6% phenol. The charcoal adsorbate was stirred with 90% aqueous phenol (170 kg.) for 0·5 hr., the mixture filtered and the charcoal re-extracted with a second portion of 90% phenol. The combined phenol eluates were stirred with ether (1400 l.) and water (120 l.). After allowing to settle overnight, the aqueous layer was run off and two further extractions made, each with 60 l. of water.

The combined aqueous extracts were adjusted to pH 4 with HCl and filtered. The filtrate was concentrated under reduced pressure to 15 l. This concentrate usually contained about 200 μg. of vitamin B_{12} activity and about 100 mg. of total solids per ml.

When treatment with nitrous acid was included it was carried out at this stage by adding $NaNO_2$ (0·75 kg.) and 5N-HCl to pH 3·8–4·2. After standing for 0·5 hr. and re-adjustment of pH if necessary, the solution was filtered. To remove some impurities the solution was extracted with 3×1 vol. of *n*-butanol, maintaining the volume and pH of the aqueous phase by addition of water and HCl as necessary. The aqueous layer was adjusted to pH 7·3–7·7 with 5N-NaOH, filtered and extracted with 3×1 vol. of phenol-butanol (1:2 by volume). The combined solvent extracts were washed with 2×5 l. portions of water.

The phenol-butanol extract was stirred with ether (180 l.) and water (15 l.). After separating the aqueous layer, two further extractions were made, each with 7·5 l. of water. The combined aqueous extracts were re-extracted with ether and concentrated under reduced pressure to one-third the original volume.

When treatment with nitrous acid was included, it was repeated at this stage by adding $NaNO_2$ (5 g./l. of concentrate) and heating to 70–80° for 5 min., adding 5N-HCl as necessary to maintain pH 3·8–4·2.

Some impurities were removed by adding $(NH_4)_2SO_4$ (200 g./l.) and filtering through a kieselguhr bed. The filtrate was mixed with kieselguhr (20 g./l.) and additional $(NH_4)_2SO_4$ (300 g./l.), and filtered after standing for a short time. The precipitate was extracted with ethanol (1 l.) and the extraction repeated until no more colour was removed. The bulked extracts were stirred with kieselguhr (80 g.) while adding ether (3 vol.), to precipitate the active material. The precipitate was filtered off and extracted with small portions of water until the extracts were colourless.

At this stage the total solids was determined. Charcoal (Sutcliffe Speakman no. 5) and kieselguhr, each equal to four times the weight of solids in the concentrate, were slurried with water and made into a bed about 1 cm. deep on a Büchner funnel. The concentrate was filtered slowly through this bed, followed by 10% aqueous *n*-propanol at the rate of 25 l./kg. of charcoal to remove impurities. The adsorbate was then eluted with 50% aqueous propanol at the rate of 15 l./kg. of charcoal. The propanol eluate was concentrated under reduced pressure to remove the propanol and reduce the volume to about 250 ml.

Alumina (200 g., Spence Grade 0) was well washed with very dilute HCl at pH 3·8 and then with water. A short chromatogram column was prepared with this alumina and the concentrate was percolated through the column, followed by distilled water until the effluent was pale pink in colour. Brown impurities were retained on the alumina. This effluent plus washings was adjusted to pH 4 and stirred with granular charcoal (Sutcliffe Speakman no. 207 B), adding the charcoal until the supernatant was colourless. The adsorbate was well washed with water, then eluted with 50% aqueous *n*-propanol until no more red colour was extracted. The eluate was concentrated to a syrup and dissolved in *n*-butanol, adding water if necessary so that the solution was just saturated with water.

A kieselguhr partition chromatogram was prepared as follows: kieselguhr (Hyflo Super-Cel) was washed with dilute HCl, then with water and dried at 150°. The kieselguhr was stirred with about six times its weight of water-saturated *n*-butanol, while half its weight of water was added slowly with vigorous stirring. The slurry was poured into a chromatogram tube and the kieselguhr column compacted by applying gentle air pressure to the top of the column and running through additional water-saturated butanol if necessary. Columns varying from 1 to 6 in. in diameter were used for various preparations. We found it desirable to allow about 1 kg. of kieselguhr for each 0·5 g. of vitamin B_{12} activity, usually associated with about 5 g. of solids. The concentrate in wet butanol was then added and the chromatogram was developed with water-saturated butanol.

Three red bands appeared on the column (sometimes four), due to vitamin B_{12c} (*R* value about 0·4), vitamin B_{12} itself (*R* value about 0·27) and vitamin B_{12b} (*R* value about 0·05). The *R* values differed somewhat from one run to another, probably owing to variation in the tightness of packing of the moist kieselguhr on the column, but the relative *R* values did not change significantly. The faster zones were usually developed right through the column. The various red fractions generally crystallized fairly readily after concentrating, precipitating with excess of acetone, redissolving in water and adding enough acetone to give slight turbidity. If necessary they were first purified further by adsorption on charcoal.

The charcoal eluate usually contained about 35% of the vitamin B_{12} activity of the fermentation liquor. When the HNO_2 treatments were included, the yield after chromatography on alumina had fallen to about 11%. The yield of crystalline material was about 6%, of which 4 or 5% was vitamin B_{12c} and 1 or 2% vitamin B_{12} itself.

Paper chromatography

Partition chromatography on paper (Whatman no. 4) was used to distinguish vitamins B_{12b} and B_{12c}. The usual descending method of development was used with water-saturated *sec.*-butanol. Sufficient material was used (0·1 mg. or more) to give clearly visible spots. The R_F values were about 0·25 for vitamin B_{12c} and 0·05 for vitamin B_{12b} (the R_F for vitamin B_{12} is 0·2 approximately). The chromatograms were usually developed overnight when the solvent front ran off the bottom of the paper. Control spots of the pure substances were used for identification.

Preparation of radioactive vitamin B_{12b}

Radioactive vitamin B_{12} (0·1 mg. containing 1·55 μc. of ^{60}Co) (Lester Smith, Hockenhull & Quilter, 1952) was added to ordinary vitamin B_{12} (13·4 mg.) dissolved in water (50 ml.). The solution was acidified to about pH 4 and illuminated with two 500 W. lamps for several hours while a stream of N_2 was bubbled through the solution. The apparatus described by Boxer & Rickards (1951) was used and the treatment was continued until HCN could no longer be detected in the effluent gas.

To remove acid the solution was run through a column of FF/X4 resin (5 g.) previously washed with a NaOH solution followed by water. The filtrate and washings were concentrated under reduced pressure to about 0·5 ml., then excess acetone was added to induce crystallization. The product was recrystallized from aqueous acetone yielding vitamin B_{12b} with a specific activity of 0·115 μc./mg.

Preparation of radioactive 'vitamin B_{12d}'

Vitamin B_{12} containing a little [^{60}Co]vitamin B_{12} was converted into vitamin B_{12c} by treatment with sulphite followed by nitrite (Lester Smith, 1952*b*). The radioactive vitamin B_{12c} (5 mg.) was dissolved in water (1 ml.) and treated with sulphamic acid (2·5 mg.) during 1 hr. at room temperature. The solution was extracted with 1 ml. of phenol and the extract washed with two portions of 0·1 N-NaOH (2 ml.), then with water, to prepare the free base. The phenolic solution was treated with acetone (0·5 ml.) and ether (10 ml.) and centrifuged. The red precipitate was dissolved in a few drops of water and crystallized by adding excess acetone. The resulting 'vitamin B_{12d}' had a specific activity of 1·4 μc./mg.

Partition chromatography of radioactive vitamin B_{12b} and 'vitamin B_{12d}'

The solvent was 15% (w/v) phenol in *n*-butanol just saturated with water. The solvent was shaken with one-tenth its volume of 2% phosphate buffer at pH 7. Acid-washed kieselguhr (Hyflo Super-Cel, 60 g.) was suspended in the solvent mixture (360 ml.) and 30 ml. of the aqueous layer was added with vigorous stirring. Part of the slurry was poured into an 18 mm. diameter tube to form a column about 25 cm. in height after compacting by slight air pressure.

1 mg. each of radioactive vitamin B_{12b} and ordinary 'vitamin B_{12d}' were dissolved in about 2 ml. of the solvent and added to the column. The chromatogram was developed until the red band nearly reached the bottom. The column was then sucked dry and extruded and the red band was cut into roughly equal top, middle and bottom segments. Each was eluted with 50% ethanol and the solution concentrated under reduced pressure and made up to 10 ml. Radioactivity was measured in a solution counter and the vitamin B_{12b} concentration was estimated colorimetrically. The specific activities of the materials from the three segments were respectively 340, 360 and 360 counts/min./mg.

Partition chromatography of radioactive 'vitamin B_{12d}' and vitamin B_{12b}

The chromatogram was prepared as described above. The solute consisted of 1 mg. of radioactive 'vitamin B_{12d}' and 1·5 mg. of vitamin B_{12b}. The red band was cut into two segments; the specific activity of the material from each was 6700 counts/min./mg.

SUMMARY

1. Three red, crystalline, microbiologically and clinically active B_{12} vitamins have been isolated from *Streptomyces griseus* fermentations, namely vitamin B_{12} itself, vitamin B_{12b} and vitamin B_{12c}.

2. Vitamin B_{12c} results from the treatment of vitamin B_{12b} with nitrous acid and it contains nitrite in the molecule.

3. Removal of this nitrite group, by treatment with sulphamic acid or distillation of an acidified solution, yields vitamin B_{12b} again.

4. Absorption spectra and other physical constants have been measured.

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B_{12} Vitamins (Cobalamins)

2. NEUTRAL, BASIC AND ACIDIC COBALAMINS

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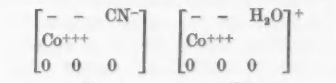
(*Received* 26 *January* 1952)

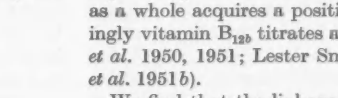
Since 1949 we have been studying the reactions of the B_{12} vitamins with sulphur dioxide, with cyanide, and with other reagents. Publication was delayed, and independent work in the same field has meanwhile been published (Fricke, Lanius, DeRose, Lapidus & Frost, 1950; Veer, Edelhausen, Wijmenga & Lens, 1950; Brink, Kuehl & Folkers, 1950; Kaczka, Wolf, Kuehl & Folkers, 1950; Beaven, Holiday, Johnson, Ellis & Petrow, 1950; Conn, Norman & Wartman, 1951; Kaczka *et al.* 1950; Kaczka, Wolf, Kuehl & Folkers, 1951).

It has become clear that the B_{12} vitamins readily form adducts with a variety of reagents in a similar manner to the haem pigments. In the light of our own and other investigations, we can now offer a more general interpretation of these phenomena than has previously been given.

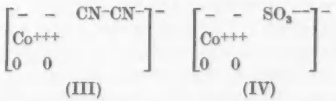
It appears that there are three classes of adducts: neutral, basic and acidic. Their nature can be illustrated by the partial structures (I), (II), (III) and



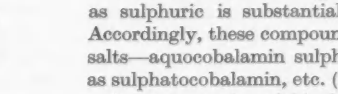
(I)



(II)



(III)



(IV)

(IV) (see Buhs, Newstead & Trenner, 1951). In vitamin B_{12} itself (Cyanocobalamin, I) the trivalent cobalt atom is linked to three neutral groups (0 0 0) and to three negative groups (– – CN^-), making the molecule as a whole neutral. The cyanide ion can be replaced by others with strong co-ordinating tendencies, to yield other neutral cobalamins; thus nitrite and thiocyanate give respectively nitritocobalamin (vitamin B_{12c}) and thiocyanatocobalamin. The compounds in this series are comparatively stable and can readily be obtained crystalline.

Photolysis or treatment with hydrogen and a catalyst or with sulphurous acid or certain other reducing agents, displaces the cyanide ion from vitamin B_{12} to yield vitamin B_{12b} (vitamin B_{12c} appears to be a dehydration product: see Kaczka, Wolf & Folkers, 1949; Kaczka, Denkewalter, Holland & Folkers, 1951; Cooley *et al.* 1951*a*). Vitamin B_{12b} appears to be an equilibrium mixture of hydroxocobalamin and aquocobalamin (II), the latter being the favoured form except in alkaline solution. As in vitamin B_{12b} the negative ion is replaced by the neutral molecule H_2O, the molecule as a whole acquires a positive charge, and accordingly vitamin B_{12b} titrates as a base (Kaczka, Wolf *et al.* 1950, 1951; Lester Smith *et al.* 1951; Cooley *et al.* 1951*b*).

We find that the linkage with strong acids such as sulphuric is substantially ionic in character. Accordingly, these compounds are better named as salts—aquocobalamin sulphate, etc.—rather than as sulphatocobalamin, etc. (Lester Smith, 1952).

Another member of this class of basic B_{12} vitamins is the substance called cobalichrome by Cooley *et al.* (1951*b*), in which NH_3 replaces the H_2O in aquocobalamin. The unstable adduct with elemental iodine also belongs presumably to this category.

Adducts belonging to the acidic class arise from the addition of two acidic ions to the cobalamin residue. It has been suggested that, where both groups are CN^-, the second displaces the benziminazole N-3 from co-ordination with the cobalt (Beaven *et al.* 1950). This is indicated in (III) by omission of one of the 0 groups. In any event, this second acidic group confers a net negative charge on the molecule, which accordingly behaves as a weak acid (Lester Smith *et al.* 1951). The CN^- groups can be replaced by two other suitable groups, which need not be the same, so that mixed double adducts can exist. The acidic cobalamins are much less stable than the neutral and basic ones. It is almost impossible to isolate them in crystalline form because conversion to the double adduct is only complete in presence of excess of the ion concerned. The behaviour of the sulphite adduct clearly places it in this class (IV); evidently the bivalent sulphite ion has the same effect as the two univalent ions present in the double adducts.

The existence of these compounds can only be inferred from the properties of their solutions. Conn *et al.* (1951) have provided polarographic evidence for the uptake of additional cyanide by vitamin B_{12}. The striking colour change to purple and the corresponding considerable changes in the absorption spectrum provide further evidence. The changes in partition coefficients between water and suitable solvents caused by addition of cyanide afford further proof of combination, while the variation in partition coefficient with pH indicates the acidic character of the complex. Adducting agents other than cyanide cause similar, though sometimes less marked, changes in the behaviour of the original cobalamin.

RESULTS

The experimental results that have contributed to our understanding of the numerous cobalamins can conveniently be grouped together under the various techniques employed, namely measurements of partition coefficients and of absorption spectra, and radioactive tracer studies.

Partition data

Partition coefficients for a range of cobalamins between benzyl alcohol and dilute aqueous buffer solutions are presented in Table 1. Comparison with the values given by Kaczka, Wolf *et al.* (1950, 1951) is not possible because they used a different solvent mixture and also because they did not specify pH values, which are sometimes important.

Table 1. *Partition coefficients of various cobalamins and the effect of* pH *on partition*

$$\left(\text{Partition coefficients: } \frac{\text{Concn. in benzyl alcohol}}{\text{Concn. in water}}.\right)$$

pH	9·5	8·5	8	6·5	5·8	5	4	3	2
Vitamin B_{12} (cyanocobalamin)	0·8	—	—	0·8	—	—	0·8	—	0·7
Dicyanocobalamin	0·75	—	0·9	—	—	1·9	—	2·9	—
Vitamin B_{12b} (aquocobalamin)	0·4	—	—	—	—	—	0·045	—	0·04
Vitamin B_{12c} (nitritocobalamin)	0·6	—	—	—	—	1·2	1·3	—	—
Thiocyanatocobalamin	0·5	—	0·65	0·85	—	—	—	0·7	—
Thiocyanatocobalamin + trace KCNS	—	—	—	—	1·15	—	1·7	—	2·5
Thiocyanatocobalamin in 5% NH_4CNS	—	0·25	0·4	0·8	—	—	2·1	—	12·5
Vitamin B_{12} + trace KCNS	0·65	—	0·65	—	0·85	—	0·95	—	1·4
Vitamin B_{12} in 5% NH_4CNS	—	—	0·2	0·3	—	—	0·5	—	3·5
Vitamin $B_{12b} + I_2$	—	—	—	2	—	6·5	—	—	3
Vitamin $B_{12} + I_2$	—	—	—	0·7	—	1·15	—	1·2	—
Sulphitocobalamin	Too low to measure								

Neutral cobalamins. As may be seen from Table 1 the partition coefficient for vitamin B_{12} is independent of pH and that of two other neutral cobalamins is nearly so. The concentrations in the two phases are similar for these compounds, whereas vitamin B_{12b} strongly favours the aqueous phase, especially at acid pH values.

Iodocobalamin. On adding a trace of iodine to a neutral or acid solution of vitamin B_{12b}, most of the colour passed into benzyl alcohol; it passed back into the aqueous phase on shaking with a little thiosulphate or *iso*propenyl acetate—substances that react readily with iodine. Some iodine was taken up on gradual addition to an aqueous solution of vitamin B_{12b}; however, when the amount reached about half an atom of iodine per molecule, tests for iodine became positive (starch test and extraction by carbon tetrachloride). Combination with iodine is evidently loose and reversible: it was not possible to prepare a pure crystalline specimen of the adduct. The compound should presumably be called 'iodocobalamin', although in the nomenclature proposed by Kaczka and his colleagues this would signify the different compound of vitamin B_{12b} with iodide ion, which we regard as aquocobalamin iodide.

Dithiocyanatocobalamin. The partition behaviour of the acidic adducts is best typified by that of the dithiocyanate adduct. Vitamin B_{12b} combines with 1 molecule of thiocyanate to yield the stable crystallizable thiocyanatocobalamin. Addition of excess

thiocyanate, however, causes changes in the spectrum (see below) and the partition coefficient: distribution into benzyl alcohol is markedly depressed in alkaline and enhanced in acid solution.

Dicyanocobalamin. The purple cyanide adduct behaves differently in acid solutions because these complexes can only persist in presence of excess of the ion concerned, and hydrocyanic acid is very feebly ionized. If an alkaline solution of the adduct is acidified and shaken immediately and briefly with benzyl alcohol, the purple compound is readily extracted (distribution coefficient $K = 2{\cdot}9$ at pH 3). On standing for a few minutes, however, the purple colour changes to red in both layers, and if the mixture is then shaken again, much of the colour passes back into the aqueous layer ($K = 0{\cdot}8$ for the resulting vitamin B_{12}).

Further evidence for the acidic character of these adducts is provided by the formation of insoluble salts on adding soluble salts of suitable metals. Examples are the purple silver and zinc salts of dicyanocobalamin and the red silver salt of dithiocyanatocobalamin.

Thiocyanatocyanocobalamin. The addition of an excess of thiocyanate to vitamin B_{12} solutions greatly assisted extraction from acid and hindered extraction from alkaline solutions, suggesting the formation of a mixed adduct (Table 1). The values of the partition coefficients, the absorption spectrum and tracer studies all combined to show that these results were not due merely to displacement of the cyanide ion by two thiocyanate ions, although this did occur to some extent.

Interconvertibility of cobalamins

Vitamin B_{12b} reacts very quickly with sulphite to give the adduct. Reaction with vitamin B_{12} itself is slow near neutrality, requiring about 1 day for completion, but is more rapid in acid solution. On aeration or evaporation of a solution of sulphitocobalamin, conversion to vitamin B_{12b} occurs, with loss of sulphur dioxide or oxidation to sulphate. Vitamin B_{12c} (nitritocobalamin) is also slowly converted into sulphitocobalamin on treatment with sulphite. Conversely, sulphitocobalamin is converted into nitritocobalamin by treatment with excess of nitrite. All the cobalamins are converted into vitamin B_{12} itself on treatment with cyanide.

Absorption spectra

On the addition of 1 equivalent of various acid ions to vitamin B_{12b} which is in the form of aquocobalamin there is little or no change in the principal ultraviolet or visible absorption maxima, except with cyanide to yield vitamin B_{12} itself. Vitamin B_{12} is the only one of these compounds to have a bright ruby-red colour in the crystalline state: the others are all very dark red in colour.

The addition to vitamin B_{12b} of two cyanide or thiocyanate ions or of a single sulphite ion does cause spectral shifts, but again the effect is largest with cyanide. Dithiocyanatocobalamin has an absorption maximum in the ultraviolet at 257 mμ. and a flat maximum in the visible at 510–540 mμ. Thiocyanatocyanocobalamin has the principal maxima at the same wavelengths as vitamin B_{12}. Dicyanocobalamin has maxima at 368 and 580 mμ.; sulphitocobalamin at 364 and 515 mμ.

Tracer experiments

Radioactive isotopes of cobalt, phosphorus, carbon, sulphur and sodium have all been used in the course of our studies of the structure and stability of the cobalamins.

[^{14}CN]*Cyanocobalamin.* We have prepared [^{14}CN] cyanocobalamin (Boxer, Rickards, Rosenblum & Woodbury, 1951) by treatment of vitamin B_{12b} in strong aqueous solution with K^{14}CN and the subsequent addition of acetone to induce crystallization; the specific activity (0·73 μc./mg.) agreed with the calculation for one cyanide group per molecule. Alternatively, vitamin B_{12} itself could equally well be used, since facile exchange between —CN$^-$ and ^{14}CN$^-$ occurs in alkaline solution.

Dicyanocobalamin. Potassium cyanide in excess (or, better, ethylamine cyanide, which is soluble in acetone) was added to strong aqueous solutions of vitamin B_{12}, followed by excess acetone. A purple microcrystalline powder was slowly deposited, sometimes mixed with larger crystals of vitamin B_{12}. From radioactive cyanide and vitamin B_{12b} we were never able to obtain a preparation having a specific activity corresponding with two cyano groups per molecule: the best appeared to contain about equal parts of di- and mono-cyanocobalamin.

The purple compound was readily extracted from alkaline aqueous solutions with phenol-benzene mixtures or benzyl alcohol, and we attempted to determine its cyanide content by radioactivity measurements on these extracts. This approach failed because inorganic cyanide was also extracted and could not be removed fully by two alkali washes.

^{24}Na *experiments.* In a similar fashion we tried to confirm the acidic character of this and other adducts by extracting into organic solvents their salts with radioactive ^{24}Na. These experiments were also frustrated by extraction of sodium cyanide into benzyl alcohol and of sodium sulphite into phenol. However, after making allowance for the rather large 'blank', it did appear that the sulphite adduct forms a salt with one sodium ion. We could also show clearly, with the help of ^{24}Na, that nitritocobalamin (vitamin B_{12c}) forms no such salt.

Aquocobalamin sulphate. Vitamin B_{12b} was freed from acid ions by passing its aqueous solution

through a column of a strongly basic ion-exchange resin, or more simply by shaking a phenolic solution with aqueous sodium hydroxide. A strong aqueous solution of the vitamin B_{12b} was acidified with $H_2{}^{35}SO_4$, and acetone was added to cause crystallization. The specific activity of the product showed that it contained 1 equivalent of sulphuric acid, i.e. $\frac{1}{2}H_2{}^{35}SO_4$, per molecule. The linkage appeared to be ionic in character. The radioactive sulphate exchanged immediately with sodium sulphate in dilute neutral aqueous solution. It was also completely removed on shaking a phenolic solution of the aquocobalamin sulphate with dilute sodium hydroxide for a few seconds.

In contrast, the specific activity of vitamin B_{12} containing ^{14}CN was undiminished by this treatment, and exchange with neutral cyanide was slow (see later). However, tracer experiments confirm other evidence that the stability of the Co—CN bond is unique. Thiocyanatocobalamin, for example, behaved more like the sulphate salt: washing a phenolic solution with dilute sodium hydroxide removed 85% of the radioactivity from [^{35}S]thiocyanatocobalamin; exchange with dilute neutral thiocyanate took place to the extent of 45% within half a minute.

Sulphitocobalamin. Attempts to prepare a pure specimen of sulphitocobalamin were unsuccessful, partly because of facile atmospheric oxidation to aquocobalamin sulphate, already noted by Kaczka, Wolf *et al.* (1951). Instability of the adduct also prevented efforts to determine its sulphite content radiometrically by partition methods.

[^{35}S]*Thiocyanatocobalamin.* Thiocyanatocobalamin was prepared by the method of Buhs *et al.* (1951), but using KCN^{35}S. The specific activity agreed within experimental error with that calculated for one sulphur atom per molecule. Solubility of thiocyanate in benzyl alcohol prevented us from establishing, by tracer methods, the existence of dithiocyanatocobalamin, inferred from partition and spectroscopic evidence.

Exchange experiments

The results given in Table 2 show that at pH 10 exchange between the cyano group of vitamin B_{12} and potassium cyanide was in 1 hr. almost complete in dilute solution and complete in stronger solutions. At pH 7 exchange was much slower and still slower at pH 3.

Table 2. *Exchange between vitamin B_{12} and cyanide using* ^{14}C

Components		Concentrations (mg./ml.) Cobalamin	KCN	pH	Time	Exchange (%)
[^{14}CN]Cyanocobalamin	KCN	0·07	0·065	10	10 min.	60
[^{14}CN]Cyanocobalamin	KCN	0·03	0·50	10	1 hr.	97
Cyanocobalamin	K^{14}CN	6·0	1·6	10	1 hr.	100
[^{14}CN]Cyanocobalamin	KCN	0·063	0·063	7	1 hr.	30
[^{14}CN]Cyanocobalamin	KCN	0·035	0·04	7	50 hr.	98
[^{14}CN]Cyanocobalamin	KCN	0·063	0·063	3	1 hr. dark	5
[^{14}CN]Cyanocobalamin	KCN	0·063	0·063	3	0·25 hr. light + 0·75 hr. dark	40
[^{14}CN]Cyanocobalamin	KCN	0·063	0·063	3	0·5 hr. light + 0·5 hr. dark	77
[^{14}CN]Cyanocobalamin	KCN	0·063	0·063	3	65 hr. dark	99
[^{14}CN]Cyanocobalamin	KCN	0·035	0·04	3	50 hr. dark	65
Cyanocobalamin	K^{14}CN	6·0	1·6	3	1 hr.	25

Only 5% exchange occurred in 1 hr. at pH 3 in the dark, but exchange was much greater if the solution was exposed to the light of a 500 W. bulb for part of the time. This effect of light was to be expected from the observation of Veer *et al.* (1950) that light causes conversion of vitamin B_{12} into vitamin B_{12b}.

The rapid exchange in alkaline solution does not imply that the Co—CN bond is ionic in character or that it is broken even momentarily, because exchange almost certainly occurs via the labile dicyanocobalamin; this implies that the two CN groups are equivalent. This exchange mechanism would operate much less effectively in neutral solution and scarcely at all at acid pH, because dicyanocobalamin is only stable in alkaline solutions.

It was of interest to determine whether the cyano group of vitamin B_{12} could be exchanged for other acid groups, such as thiocyanate. The results were similar, whether we used ordinary vitamin B_{12} with KCN^{35}S or [^{14}CN]cyanocobalamin with ordinary KCNS. Relatively high concentrations were used and a reaction time of only 10 min. Under these conditions, as Table 3 shows, about 30% exchange occurred in acid or alkaline solutions, but little in neutral solution. It appears that equilibrium is reached rapidly, but that even an overwhelming excess of thiocyanate displaces only a small proportion of the co-ordinated cyanide, owing to the much stronger attachment of the latter to the cobalt. A similar experiment with [^{14}CN]cyanocobalamin in 5% (w/v) sodium nitrite at pH 10 showed only 5% exchange in 10 min.

Table 3. *Exchange between vitamin B_{12} and thiocyanate measured using ^{35}S*

Components		Concentrations (mg./ml.) Cobalamin	Salt	pH	Time	Exchange (%)
Cyanocobalamin	$KCN^{35}S$	1·5	10	2·5	10 min. dark	30
Cyanocobalamin	$KCN^{35}S$	1·5	10	2·5	10 min. light	32
[^{14}CN]Cyanocobalamin	KCNS	0·3	50	7	10 min.	<5
[^{14}CN]Cyanocobalamin	KCNS	0·3	5	7	10 min.	<5
[^{14}CN]Cyanocobalamin	KCNS	0·3	0·5	7	10 min.	<5
[^{14}CN]Cyanocobalamin	NH_4CNS	0·25	50	7	10 min.	5
Cyanocobalamin	$KCN^{35}S$	1·5	10	8·5	10 min. dark	33
Cyanocobalamin	$KCN^{35}S$	1·5	10	8·5	10 min. light	29
Cyanocobalamin	$KCN^{35}S$	3	30	10	10 min.	26
[^{14}CN]Cyanocobalamin	KCNS	0·7	50	9·5	10 min. dark	26
[^{14}CN]Cyanocobalamin	NH_4CNS	0·25	0·5	8·5	10 min.	5
[^{14}CN]Cyanocobalamin	NH_4CNS	1·1	50	8	1 hr. dark	10
[^{35}S]Thiocyanatocobalamin	NH_4CNS	0·15	1	7	Immediate	45
[^{35}S]Thiocyanatocobalamin	NaOH	0·3	0·4	8·5	Immediate	85
[^{35}S]Thiocyanatocobalamin	NaOH	0·2	0·4	8·5	Immediate	85

EXPERIMENTAL

Partition coefficients. Partition coefficients were measured at room temperature by shaking an aqueous solution of the cobalamin in dilute phosphate buffer with benzyl alcohol, centrifuging to separate the layers and then diluting measured volumes of the two layers with ethanol to suitable strengths for colorimetric assay. The intensity of colour was the same whether the solvent was water, aqueous ethanol or a mixture of benzyl alcohol and ethanol. The pH of the aqueous phase was checked before dilution. Volumes of the two phases were chosen such that the cobalamin was distributed between them in roughly equal amounts. About 2 mg. of the cobalamin in a total volume of about 10 ml. were used for each measurement.

Preparation of [^{14}CN]*cyanocobalamin.* Vitamin B_{12b} (9·5 mg.), prepared by treatment of vitamin B_{12} with H_2 in presence of platinum oxide catalyst (Kaczka *et al.* 1949) was dissolved in water (0·3 ml.) containing $K^{14}CN$ (0·5 mg.: 7·5 μc.). After 1 hr. at room temperature 2 ml. water and 1 drop of glacial acetic acid were added, and the mixture was extracted with 0·5 ml. of benzene-phenol mixture (2:1). The solvent layer was washed with two portions of about 1 ml. of water, then enough acetone was added to precipitate all the coloured material. The precipitate was collected by centrifuging, washed with acetone, and dissolved in 1 ml. of 70% acetone. The solution was centrifuged to remove a trace of suspended matter, then further acetone was added to the point of incipient turbidity. The crystals that separated in a few hours were redissolved in a few drops of water and again crystallized by adding acetone. The yield was 5 mg. (0·73 μc./mg.) of anhydrous vitamin B_{12}.

Preparation of [^{14}CN]*cyanocobalamin by exchange.* Vitamin B_{12} (8·5 mg.) was dissolved in 1 ml. of water containing $K^{14}CN$ (1·65 mg.: 25 μc.). After 1 hr. at room temperature, 1 drop of glacial acetic acid was added and the solution was extracted with 1 ml. of benzene-phenol mixture (2:1). The solvent layer was washed with 2 ml. portions of water, 0·01 N-NaOH and water, successively, and then mixed with enough ether-acetone mixture (1:1) to precipitate nearly all the coloured material. This was centrifuged down, washed with acetone, crystallized and recrystallized from aqueous acetone. The specific activity was 0·62 μc./mg. anhydrous vitamin B_{12}, compared with the value of 0·61 μc./mg. calculated on the basis of complete exchange.

Preparation of $KCN^{35}S$. Radioactive KCNS was prepared by fusing KCN (0·5 g.) with ^{35}S (0·25 g.: 20 μc.). The melt was extracted with water and the $KCN^{35}S$ purified by crystallizing and recrystallizing from water; this was difficult on the small scale owing to the high solubility of the salt. The yield was 350 mg. of material, 95% pure by titration against 0·1 N-$AgNO_3$. The activity was 3200 counts/min./mg. $KCN^{35}S$.

Cyanide exchange experiments. The cobalamin in dilute buffer solution at the desired pH was mixed with KCN solution (one or other component being labelled with ^{14}C) in a total volume of 10 ml. The concentrations are listed in Table 2. After the desired period at room temperature, the solution was acidified with acetic acid (if alkaline) and the vitamin B_{12} was extracted with 2 ml. of liquefied phenol. After being washed twice with water (5 ml.), the extract was shaken vigorously with a few drops of water and 10 vol. of ether. Nearly all the colour was thus transferred to the small volume of water, which was separated and diluted with acetone until just turbid (about 10 vol.). The resulting crystals were rinsed with acetone, then redissolved in water. The vitamin B_{12} concentration was estimated colorimetrically, and the radioactivity as described below.

Thiocyanate exchange experiments. The experiments involved either loss of radioactivity from [^{14}CN]cyanocobalamin on partial conversion to thiocyanatocobalamin, or gain of radioactivity in the same reaction between normal cyanocobalamin and $KCN^{35}S$. They were performed in the same manner as those involving cyanide exchange, except that the volume of the reaction mixture was 0·5–2 ml. at higher concentrations, as listed in Table 3. Table 3 also includes results for reactions between [^{35}S]thiocyanatocobalamin and KCNS or NaOH, for which the same experimental procedure was used.

Radioactivity measurements. The crystalline preparations were dissolved in a suitable volume of water (1·5–10 ml.). The cobalamin concentration was determined with the Spekker photoelectric colorimeter, either directly on this solution or on a dilution, as appropriate.

The radioactivity was measured with a thin end-window Geiger-Müller counter on 0·5 ml. of the solution dried on a nickel planchette. Loss of radioactive cyanide or thiocyanate was prevented by adding excess $AgNO_3$ before drying and by using a hotplate in place of the more usual infrared lamp, which could cause some photolytic decomposition.

SUMMARY

1. The structures and stabilities of a series of cobalamins have been studied, mainly by tracer techniques and by measurements of absorption spectra and partition coefficients.

2. It has been shown that, besides the neutral and basic classes of cobalamins, there is a third class of acidic cobalamins. They contain two acidic ions, which need not be the same, or one divalent ion like sulphite. They are unstable and only persist in solutions containing excess of the co-ordinating ion.

3. Using radioactive carbon and sulphur it was shown that the cyano group in vitamin B_{12} exchanges with inorganic cyanide, rapidly in alkaline solution but very slowly in acid. In high concentrations of thiocyanate in acid or alkaline, but not in neutral solutions, the cyano group is partly displaced by thiocyanate.

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Studies on the Composition of Horse Oil

2. THE COMPONENT FATTY ACIDS OF LIPIDS FROM FATTY TISSUES, MUSCLE AND LIVER

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In part 1 of this series (Brooker & Shorland, 1950) it was shown that the fats of pasture-fed animals differed considerably from species to species in fatty acid composition. Whereas linolenic (octadecatrienoic) acid, the chief component of the pasture fatty acids, comprised about 17 % of the depot fat of horses, it was found only in traces in the fats of pasture-fed sheep and oxen. Horse fats differed further from those of sheep and oxen by having greater proportions of hexadecenoic acid and smaller amounts of stearic acid. These results have since been generally confirmed by Gupta & Hilditch (1951). In addition, Bruce & Shorland (1951) have shown in a preliminary communication that, as observed for other animals (cf. Hilditch & Shorland, 1937), the liver phospholipins of the horse contained more stearic and highly unsaturated $C_{20\text{-}22}$ acids, but less palmitic and hexadecenoic acid, than did the liver glycerides. It was found, however, that although the respective contents of total C_{18} unsaturated acids and of oleic acid of the phospholipin and glyceride fractions of the horse liver were similar, the phospholipin contained about three times as much octadecadienoic acid but only one-

Table 1. *Characteristics of horse lipids used in this investigation*

	Lipid in tissues (%)	P (%)	N (%)	Unsaponifiable (%)	Cholesterol in lipid: Combined (%)	Cholesterol in lipid: Free (%)
			FAT HORSES			
Fatty tissues (whole fraction)	93·2	Trace	Trace	0·53	—	—
Muscle glycerides	9·3	0·21	—	2·73	—	—
Muscle phospholipins	0·3	3·93	—	—	—	—
Liver glycerides	2·7	0·53	0·44	18·5	1·40	8·65
Liver phospholipins	3·3	3·80	1·89	—	—	—
			LEAN HORSES			
Fatty tissues (whole fraction)	80·1	Trace	Trace	0·20	—	—
Muscle glycerides	2·9	0·36	—	6·10	—	—
Muscle phospholipins	0·3	3·79	2·13	—	—	—
Liver glycerides	1·2	0·50	0·38	31·4	5·08	17·52
Liver phospholipins	3·3	3·58	1·77	—	—	—

sixth the amount of linolenic acid present in the glyceride. Such marked differences in composition between the C_{18} unsaturated acids of the glycerides and phospholipins of the same animal do not appear to have been previously recorded.

The purpose of the work now reported has been to examine the fatty acid composition of lipids from various tissues of the horse, particularly the distribution of the C_{18} unsaturated acid constituents.

EXPERIMENTAL

Preparation of material

The material was obtained as in the previous investigation (Brooker & Shorland, 1950) from horses killed at the Auckland Farmers' Freezing Company Works at Southdown, New Zealand. A batch of fifty-five horses was killed on the morning of 4 November 1947, yielding an average dressed weight of carcass of 721 lb. After chilling overnight the carcasses were used for sampling. In general, most of the animals contained insufficient readily available fatty tissue for study by the usual ester fractionation techniques. However, some 'fat' animals in contrast to the more typical 'lean' animals had a layer of external fatty tissue over the greater part of the body except for portions of the shoulder. In addition, the flare fat in the abdominal cavity was in places up to 5 cm. thick as compared with only a relatively thin layer in the lean horses. For the purpose of this study five typical lean horses and two fat horses were selected. The fatty tissues used were representative of the external fat as well as of the abdominal cavity, and for the lean horses it was necessary to take all the available material for the sample. The fat horses, however, contained more than 20 times as much readily available fat as the lean horses, so that a representative sample only was used. In the case of the muscle tissues a series of cuts was prepared comprising the silverside, tender loin, sirloin, prime rib-cut, blade and thin-flank. The tissues were finely sliced and representative portions taken to make a composite sample which was heated in boiling 95% (v/v) ethanol for 20 min. and then stored in sealed jars.

The liver samples, total weights 12·7 and 27·6 kg., taken respectively from two fat and five lean carcasses selected from another killing of sixty-eight horses (average dressed weight 734 lb.) on 7 November 1947, were treated and preserved as described above.

After removal of the ethanol and water *in vacuo* at 100° the tissues were extracted with light petroleum containing 10% ethanol (v/v). Following evaporation of the solvent on the water bath, under reduced pressure during the last stages, the lipids were taken up in approximately 10 vol. of light petroleum and washed with about 0·1 vol. of water three times to remove impurities. The aqueous layers after evaporation *in vacuo* were re-extracted with light petroleum, but yielded no appreciable amount of lipid.

Separation of phospholipins and glycerides

The separation of the phospholipins and glycerides was carried out in the usual way by dissolving the lipids as completely as possible in 9 vol. of boiling acetone and refluxing for 20 min. The mixture was then allowed to cool to room temperature (cf. Hilditch & Shorland, 1937). To remove the traces of phospholipins present in the crude glycerides, the acetone-soluble fractions were cooled to 0° in 9 vol. of the solvent, in the presence of a few drops of an ethanolic solution saturated with $CaCl_2$. After decanting the supernatant liquid the small amount of precipitate was refluxed with acetone and allowed to cool, and the acetone solution again decanted to separate the acetone-insoluble material which was then added to the phospholipin fraction. Even though these processes were carried out repeatedly, the glyceride fractions could not be freed from phospholipin. Further purification (cf. Table 2) of the liver glycerides of the fat and lean horses, which contained respectively 16·8 and 14·5% of free fatty acids (calculated as oleic), was achieved by dissolving the fractions in ether and washing with 10% (w/v) K_2CO_3 solution.

In the case of fat horses, the crude glycerides of the muscle (98·0 g.) were dissolved in light petroleum (b.p. 40–70°) and chromatographed on a column 4·0 cm. in diameter packed with a mixture (300 g.) containing equal proportions by weight of finely ground MgO and $CaCO_3$. The glyceride fraction (85·96 g.) obtained by repeated elution with light petroleum showed no detectable amount of P but contained 0·45% of free fatty acids expressed as oleic acid. The adsorbed material (11·38 g.; 1·62% P) recovered by extraction with ether after acidification of the

adsorbent with dilute HCl consisted of 56·8% free fatty acids (expressed as oleic) and phospholipins. Further treatment with acetone according to the procedure outlined above yielded an acetone-insoluble fraction (3·89 g., 3·15% P).

Ester fractionation analyses

These were carried out as previously described by Brooker & Shorland (1950). In the case of the phospholipins, however, the fractions were first submitted to ethanolysis by boiling for 3 hr. under reflux with 3 vol. of ethanol containing 1% (w/v) H_2SO_4 before saponification with ethanolic potash in the usual way.

The ester fractionation analyses of liver glycerides were based on the purified fractions (cf. Table 2) from which the unsaponifiable matter was removed by thorough extraction of the aqueous ethanolic soap solutions with ether.

Since fatty acid analyses of the crude and purified fractions of the muscle glycerides of the fat horses, based respectively on 76·8 and 134·8 g. of material, showed no appreciable differences (cf. Table 3) within the limits of the accuracy of the method, the glyceride fraction from the muscle tissue of the lean horses was not further purified, and the results, shown in Table 5, for the muscle tissue glycerides of both the fat and the lean horses were based on the unpurified fractions.

The amounts of muscle phospholipins were too small for an accurate ester fractionation analysis. Accordingly, the fatty acids were separated into 'solids' and 'liquids' as described by Brooker & Shorland (1950) and the characteristics of the fractions determined as shown in Table 4, which includes, for comparison, data obtained for the liver phospholipins.

Composition of the C_{18} unsaturated acids

The composition of the C_{18} unsaturated acids was studied separately by applying the alkali-isomerization method of Hilditch, Patel & Riley (1951) to purified concentrates of C_{18} unsaturated acids obtained during the course of ester fractionation analyses. Although the muscle phospholipins, as stated above, were not available in sufficient quantity for an accurate ester fractionation analysis, it was found practicable to prepare, by distillation of the methyl esters of the liquids, a C_{18} fraction for analyses by the alkali-isomerization method.

Table 2. *Purification of the crude liver-glyceride fractions*

Purified glyceride		Ether-soluble fractions from acidified K_2CO_3 washings: Acetone-soluble free fatty acids		Acetone-insoluble phospholipins	
% of original fraction	P (%)	% of original fraction	P (%)	% of original fraction	P (%)
FAT HORSES					
71·3	0·13	21·7	0·72	7·0	3·60
LEAN HORSES					
77·1	0·14	15·1	0·91	7·8	2·75

Table 3. *Comparison between the fatty acid composition of the crude and purified glycerides of muscle tissue of fat horses*

	Fatty acids (wt. %): Saturated					Unsaturated			
	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}	C_{14}	C_{16}	C_{18}	C_{20}
Crude glyceride	0·4	2·4	29·9	3·0	0·2	1·5 (−2·0H)	8·7 (−2·0H)	50·8 (−3·2H)	3·1 (−4·4H)
Purified glyceride	—	3·9	30·4	2·5	—	1·6 (−2·0H)	9·4 (−2·0H)	48·8 (−3·2H)	3·4 (−3·9H)

Table 4. *Characteristics of muscle and liver phospholipin fatty acid fractions*

Fraction	Solids: % of total	Saponification equivalent	Iodine value	Liquids: % of total	Saponification equivalent	Iodine value
FAT HORSES						
Acetone-insoluble from muscle lipids	32·6	284·2	19·7	67·4	289·9	168·8
Phospholipin recovered from glyceride fraction of muscle lipids	39·9	276·9	16·9	60·1	274·9	145·4
Liver phospholipins	33·4	280·2	3·8	66·6	284·1	147·8
LEAN HORSES						
Acetone-insoluble from muscle lipids	34·4	286·6	19·2	65·6	281·9	117·3
Liver phospholipins	38·3	284·6	7·1	61·7	283·4	163·3

Owing to the extreme differences between the glycerides and phospholipins in respect of their C_{18} unsaturated acid compositions (Table 6) it was found advantageous to allow for the traces of contaminating phospholipins in the glyceride fractions as outlined by Hilditch & Shorland (1937). For example, the muscle glycerides of the fat horses showed a difference between the uncorrected value (8·5%) for octadecadienoic of 1·9 units % acid content and the corrected value (6·6%), as compared with the value of about ±0·4 unit % for the standard deviation of the method.

DISCUSSION

Hilditch & Shorland (1937), in their work on the liver lipids of the ox, sheep and pig, indicated that the ester fractionation analyses of the glycerides separated from phospholipins with acetone may be somewhat in error unless a correction is made for the invariable presence of small proportions of contaminating phospholipin fatty acids. In the present work, to avoid possible errors owing to phospholipin contamination, the liver glycerides from the acetone separation were freed from all but traces of phospholipins and free fatty acids by alkali refining, while the accuracy of the fatty acid composition analyses of the muscle glycerides was confirmed by comparing the results obtained before and after purification by chromatographic adsorption.

A separate study of the C_{18} unsaturated acids by means of alkali-isomerization technique showed such extreme differences in composition between the phospholipin and glyceride fractions of the same tissues that it was found advantageous to allow for the traces of phospholipins in the glyceride fractions, and the results in Table 6 are based on the values which have been corrected according to the procedure outlined by Hilditch & Shorland (1937).

Phospholipin fractions when precipitated from acetone solution may sometimes be contaminated with glycerides of one unsaturated and two saturated acids or of three saturated acids (Hilditch & Shorland, 1937). Fortunately not more than traces of such glycerides were encountered in the present work.

Fatty acid composition of the glycerides and phospholipins

Although, from the precautions taken, as outlined above, it would appear that the glyceride fractions shown in Table 6 are strictly comparable, there still remains the possibility that the presence of cholesterol esters may affect the apparent fatty acid composition of the liver glycerides. Kelsey & Longenecker (1941) found differences in fatty acid composition between the neutral glycerides and

Table 5. *Fatty acid composition of the lipid fractions from the fatty tissues, muscle and liver of the horse.* (*Fatty acids wt.* %)

	Saturated					Unsaturated				
	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
Fat horses										
Fatty tissue glycerides	—	2·4	29·7	4·3	0·2	1·4 (−2·0H)	6·5 (−2·0H)	52·4 (−3·4H)	3·1 (−3·3H)	—
Muscle glycerides	0·4	2·4	29·9	3·0	0·2	1·5 (−2·0H)	8·7 (−2·0H)	50·8 (−3·2H)	3·1 (−4·4H)	—
Liver glycerides	—	1·7	25·2	5·3	—	1·6 (−2·0H)	8·3 (−2·0H)	52·8 (−4·5H)	3·4 (−6·2H)	1·7 (−6·0H)
Liver free fatty acids	—	—	10·5	20·6	0·5	—	9·5 (−2·0H)	48·7 (−3·8H)	5·9 (−6·0H)	4·3 (−6·0H)
Liver phospholipins	—	—	10·8	20·7	4·6	—	2·0 (−2·0H)	50·6 (−3·4H)	10·4 (−5·7H)	0·9 (−5·7H)
Lean horses										
Fatty tissue glycerides	—	3·2	26·2	5·6	0·3	0·9 (−2·0H)	4·6 (−2·0H)	56·3 (−3·5H)	2·9 (−4·1H)	—
Muscle glycerides	—	1·7	26·8	5·1	—	0·8 (−2·0H)	7·9 (−2·0H)	54·5 (−3·3H)	3·2 (−4·8H)	—
Liver glycerides	—	0·7	22·5	10·8	—	0·6 (−2·0H)	6·1 (−2·0H)	49·7 (−3·5H)	9·6 (−6·0H)	—
Liver free fatty acids			33·4			—	10·9 (−2·0H)	45·1 (−3·4H)	10·6 (−3·4H)	—
Liver phospholipins	—	—	11·1	18·2	6·5	—	3·4 (−2·0H)	48·6 (−3·3H)	9·7 (−4·5H)	2·5 (−4·5H)
Bulk sample of bone oil (Brooker & Shorland, 1950)	0·2	2·8	25·2	4·6	—	0·7 (−2·0H)	8·1 (−2·0H)	57·4 (−3·5H)	1·0 (−4·0H)	—
Australian horse oil	—	2·0	24·0	7·0	—	0·6 (−2·0H)	7·3 (−2·0H)	55·5 (−3·5H)	3·6 (−3·5H)	—

cholesterol esters of blood plasma. The amounts of combined cholesterol in the liver glycerides of the fat and lean horses, 1·40 and 5·08 % respectively, however, are not sufficient to affect appreciably the interpretation of the results.

From Table 5 it will be seen that, while all the glyceride fractions are generally similar in fatty acid composition, the liver glycerides differ from those of the muscle and fatty tissues in containing slightly less (about 4 %) palmitic acid and rather more C_{20-22} highly unsaturated acids. In addition, the liver glycerides of the lean horses are shown to contain approximately twice as much stearic acid as is present in the remaining glycerides. The absence of increased proportions of hexadecenoic acid in the liver glycerides of the horses as compared with the depot fats, distinguishes horses from sheep, oxen and pigs, where the glycerides show considerably more hexadecenoic acid than is found in the depot fats (Hilditch & Shorland, 1937). A somewhat higher proportion (3 units %) of palmitic acid is maintained in the glyceride fractions of the fat horses as compared with the corresponding fractions of the lean horses. The content of stearic acid is uniformly low (3·0–5·6 %) in all the glycerides studied except the liver glycerides of the lean horse in which it rises to 10·8 % and in which, also, the amount of C_{20} unsaturated acids increases to 9·6 %, or about three times the amount found in the remaining glycerides.

Included for comparison with the present results are the bulk sample of bone oil previously studied by Brooker & Shorland (1950), and a sample of Australian horse oil obtained by steam rendering of horses from Queensland. These analyses, together with those of previous workers summarized by Gupta & Hilditch (1951) show that the horse glycerides, irrespective of the particular fatty depot or the locality from which the sample was taken, have a generally similar fatty acid composition.

Horse fats in general resemble those of other animals in regard to their content of palmitic acid (25–30 %), but as compared with the depot fats of the ox, sheep or pig (cf. Hilditch, 1947), they contain considerably less stearic acid (3–7 % as compared with 10–30 %) but more hexadecenoic acid (4·6–8·7 % as compared with 1–3 %) and appreciable proportions (about 3 %) of C_{20-22} unsaturated acids, as compared with only trace amounts (1 % or less) found in depot fats of these animals.

The fatty acid analyses of the phospholipins accord with the previous work of Hilditch & Shorland (1937) on the liver lipids of oxen, sheep and pigs in showing, as compared with the corresponding glycerides, increased proportions of stearic acid and C_{20-22} highly unsaturated acids, together with reduced proportions of palmitic and hexadecenoic acids. The similarity in fatty acid composition between the free fatty acid and phospholipin fractions of the liver lipids, suggests that the former fraction may have originated mostly from the latter. This view is also supported by comparing the composition of the two C_{18} unsaturated acid fractions, as shown in Table 6. From previous investigations (cf. Shorland, 1948) it has been found that by taking special precautions, a low content of free fatty acids may be obtained, indicating that the amounts present in living tissue are very low indeed. Although, as previously indicated, no ester fractionation analyses were made on the muscle phospholipins because only small amounts of material were available, the data given in Table 3 suggest their fatty acid composition resembles that of the liver phospholipins.

The C_{18} unsaturated acids

Distribution of the C_{18} unsaturated acids in the glyceride and phospholipins. The close agreement between the ester fractionation analyses of all the glyceride fractions (cf. Table 5) could be taken as proof of similarity in fatty acid composition. However, it is clear from Table 6 that such is not the case, and that the C_{18} unsaturated acids of glycerides from the fatty tissues, muscle and the liver, though similar as regards their total proportions and mean unsaturations, have quite different compositions in respect to the monoene, diene and triene constituents. From Table 6 it will be seen that a similar pattern in regard to the distribution of the C_{18} unsaturated acids is followed in the corresponding glycerides of the fat and of the lean horses. The proportions of glyceride oleic acid are similar (about 60 %) in the muscle and fatty tissues, being slightly greater in the former, but are reduced in the liver to approximately half the value found in the other tissues. The proportions of glyceride diene show a threefold increase in passing from the fatty tissues to the liver, with the muscle tissues showing an intermediate value. The glyceride linolenic acid is present in greatest proportions in the liver where it comprises the main C_{18} unsaturated acid constituent. The fatty tissue glycerides are shown to contain slightly less of this constituent and the muscle glycerides least. Linolenic acid is shown to be the least variable constituent and octadecadienoic acid the most variable of the C_{18} unsaturated acid constituents of the glycerides.

According to Callow's (1935) theory, fast-growing fatty depots, as compared with those that grow more slowly, contain more of the fat synthesized by the animal and less of the fat derived by direct assimilation. In agreement with this theory the fatty tissue and muscle glycerides of the fat horses are shown to contain less of the dietary octadecadienoic and linolenic acids than the lean horses. In the liver glycerides, however, the reverse is true.

Table 6. *Composition of the* C_{18} *unsaturated acids expressed as a wt. % of the* C_{18} *unsaturated acids*

(Figures in brackets indicate wt. % expressed on the total fatty acids. (tr., trace.))

Fraction	Conjugated		Non-conjugated		Oleic (by difference)	Triene / Diene
	Diene	Triene	Diene	Triene		
			FAT HORSES			
Fatty tissue glycerides	1·0 (0·5)	tr. (tr.)	6·3 (3·3)	30·8 (16·1)	61·9 (32·5)	4·22
Muscle glycerides	0·1 (tr.)	tr. (tr.)	6·6 (3·4)	24·6 (12·5)	68·7 (34·9)	3·67
Muscle phospholipins*	0·4	0·1	66·8	8·1	24·6	0·12
Muscle phospholipins†	0·5	0·1	54·7	9·4	35·3	0·17
Liver glycerides	0·7 (0·4)	0·1 (tr.)	19·0 (10·0)	46·9 (24·8)	33·3 (17·6)	2·39
Liver free fatty acids	1·0 (0·5)	0·1 (tr.)	37·0 (18·1)	28·2 (13·7)	33·7 (16·4)	0·75
Liver phospholipins	1·2 (0·6)	0·2 (0·1)	59·5 (30·1)	7·8 (4·0)	31·3 (15·8)	0·13
			LEAN HORSES			
Fatty tissue glycerides	1·3 (0·7)	tr. (tr.)	7·0 (3·9)	35·4 (20·0)	56·3 (31·7)	4·27
Muscle glycerides	0·3 0·2	tr. tr.	11·1 (6·0)	27·0 (14·7)	61·6 (33·6)	2·37
Muscle phospholipins	2·3	0·4	40·4	23·6	33·3	0·56
Liver glycerides	1·9 (0·9)	0·4 (0·2)	16·9 (8·4)	40·4 (20·1)	40·4 (20·1)	2·17
Liver free fatty acids	2·0 (0·9)	0·3 (0·1)	34·8 (15·7)	15·1 (6·8)	47·8 (21·6)	0·42
Liver phospholipins	4·9 (2·4)	0·2 (0·1)	55·2 (26·8)	7·3 (3·5)	32·4 (15·8)	0·12

* Acetone-insoluble fraction from total lipid.

† Recovered from crude glyceride fraction.

Characteristic of the lipids of the lean horses is the somewhat higher level of conjugated diene acid, particularly in the liver phospholipins where it comprises 4·9 % of the total C_{18} unsaturated acid or approximately four times the amount present in the lipids of the fat horses.

From Table 6 it will be seen that the distribution of the C_{18} unsaturated acids follows a similar pattern in all the phospholipins studied. The main component is octadecadienoic acid (40·4–66·8 %) followed by oleic acid (24·6–40·4 %), with linolenic acid (7·3–23·6 %) as the minor constituent. As compared with the glycerides, the phospholipins are shown to contain more octadecadienoic acid, but less linolenic acid as may be judged from their respective triene/diene ratios of (2·17–4·27) and (0·12–0·56). The C_{18} unsaturated acids of the phospholipins studied show about half the amount of oleic acid found in the glycerides of the fatty tissue and the muscle, but are comparable in this respect with the liver glycerides.

The relationship between the C_{18} *triene/diene ratio of the dietary fat and of the depot fat*

The diene and triene C_{18} unsaturated acids found in the depot fats of animals are apparently not synthesized in the body, but are derived by direct assimilation from the diet (Hilditch, Lea & Pedelty, 1939; Shorland & de la Mare, 1945). In agreement with unpublished results for the C_{18} triene/diene ratio of approximately 4·0 found for typical New Zealand pasture, the C_{18} triene/diene ratios of the main depot fats of pasture-fed horses (Table 6) are shown to vary from 2·37 to 4·27. Similar values for fats of pasture-fed horses have also been obtained by other workers (Hilditch & Jasperson, 1944; Brooker & Shorland, 1950; Gupta & Hilditch, 1951).

The lower C_{18} triene/diene ratio (0·1–0·4) observed by Holmberg & Rosenqvist (1949) for the bone oils and kidney fats of horses fed oats and hay, accords with the fact that in oat lipids linoleic and not linolenic acid is the main highly unsaturated C_{18} constituent (see Hilditch, 1947). Similarly, the lower ratio of 1·02 found in the present work for the Australian horse oil could well be due to the very different types of forage plants in the Queensland tropics as compared with those in New Zealand or Great Britain.

The evidence suggests that the ratio of C_{18} triene/diene acids present in the diet remains essentially unchanged during the passage of the dietary fat through the horse, and its ultimate incorporation in the fatty depots. In contrast, pasture-fed sheep and oxen neither readily incorporate the dietary

linolenic and octadecadienoic acids in their fatty depots (Shorland, 1950) nor store such amounts of these dietary acids as are retained in the ratio in which they are present in the diet. Unpublished work in this laboratory, for example, indicates that the main fatty depots of pasture-fed sheep and oxen show a C_{18} triene/diene ratio of 0·3–0·4.

Although the main fatty depots of the horse show a C_{18} triene/diene ratio not unlike that of the pasture lipids, there is, nevertheless, some variation in the composition of the C_{18} unsaturated acids between the different fatty depots, and especially between the phospholipin and glyceride fractions (cf. Table 6). Such changes in the C_{18} triene/diene ratios could be brought about by selective transformation of octadecadienoic acid and linolenic acid, which, according to Widmer & Holman (1950), are converted by the animal to arachidonic and C_{22} hexaene acids respectively. Though some such transformation has occurred in the horse, the proportions of C_{20-22} highly unsaturated acids in the depots, as compared with those of octadecadienoic and linolenic acid, are very small indeed. Selective oxidation of the diene and triene C_{18} constituents could also be a factor in changing the C_{18} triene/diene ratio, but the presence of similar proportions of octadecadienoic acid in the depot fats of animals before and after a period of starvation (Longenecker, 1939; Hilditch & Pedelty, 1941*a*, *b*) may well indicate a comparative stability of the more highly unsaturated acids in contrast to the rapid turnover of oleic, stearic and palmitic acids postulated by Schoenheimer & Rittenberg (1936) and by Rittenberg & Schoenheimer (1937).

It is therefore concluded that selective deposition of the fatty acids during the transport of the fatty substances between the liver and the fatty depots, and vice versa, rather than selective transformation or oxidation, is the main factor responsible for the observed differences in composition of the C_{18} unsaturated acids between the fatty depots of the different tissues, and between the phospholipins and the glycerides of the same tissues of the horse.

The mechanism of fat deposition in the horse as indicated from the glyceride structure

From the principle of even distribution (Hilditch, 1947) the presence of some 60 % of linolenic acid in the glyceride fraction of pasture lipid implies the occurrence of this acid mainly in the form of dilinolenotriglycerides. In the depot fat of the horse, however, it has been shown by Gupta & Hilditch (1951) that linolenic acid is present almost entirely as a monolinolenotriglyceride. Even allowing for some enzyme action during ingestion of the pasture (cf. Shorland, 1944) with the production of some free fatty acids, the difference in glyceride structure between the dietary fat and the depot fat does not afford evidence in favour of Frazer's (1946) theory implying the absorption of some of the fat directly without conversion into free fatty acid. The evidence of the glyceride structure rather points, however, to the absorption of the dietary fat as free fatty acids, and in part, perhaps, as monoglycerides (cf. Reiser, Bryson, Carr & Kuiken, 1951) which are subsequently selectively distributed and mixed in with the fatty acids synthesized by the animal to form new glycerides in the various fatty depots of the horse.

SUMMARY

1. The fatty acid composition of the glycerides of the fatty tissues, muscle and liver of the horse as determined by ester fractionation were shown to be generally similar and to contain 25–30 % palmitic acid, 3–6 % stearic acid, 4–8 % hexadecenoic acid, 50–55 % C_{18} unsaturated acids, and minor proportions of myristic, tetradecenoic and C_{20-22} unsaturated acids.

2. A separate examination of the C_{18} unsaturated acids showed considerable differences in composition between the different glycerides. The liver glycerides, in particular, contained less oleic acid than the glycerides of the muscle and fatty tissues (33·3–40·4 as compared with 56·3–68·7 %) and the ratio C_{18} triene/diene showed a progressive diminution from 4·22–4·27 to 2·17–2·39 in passing from the glycerides of the fatty tissues to those of the muscle and liver.

3. The liver phospholipins of the horse as compared with the liver glycerides were shown, in conformity with the observations by other workers on a range of different animals, to contain more stearic acid and C_{20-22} unsaturated acids but less palmitic and hexadecenoic acid. In addition, the liver phospholipins contained more octadecadienoic acid (26·8–30·1 as compared with 8·4–10·0 %) but less linolenic acid(3·5–4·0as compared with 20·1–24·8 %) than did the liver glycerides.

4. The composition of the C_{18} unsaturated acids in the muscle phospholipins was found to be similar to that of the liver phospholipins.

5. The distribution pattern of the C_{18} unsaturated acids together with the nature of the glyceride structure in the fatty depots of pasture-fed horses compared with that of the fats in pasture suggests that the dietary fats of the horse cannot be directly absorbed as such, but that the fatty acids of the diet are mixed in with those synthesized by the animal, and selectively redistributed to form new glycerides.

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Studies in Biochemical Adaptation. The Origin of Urinary Ammonia as Indicated by the Effect of Chronic Acidosis and Alkalosis on Some Renal Enzymes in the Rat

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The origin of urinary ammonia, which is an important part of the mechanism for maintaining the acid-base equilibrium, has been the subject of much study. The early view was that it was derived from urea (Barnett & Addis, 1917; Nash & Benedict, 1921; Bollman & Mann, 1930). More recent work has shown this view to be incorrect (Van Slyke *et al.* 1943; Pitts, 1936). It is now believed that the ammonia is derived from glutamine and from other amino-acids. Whereas the kidney contains no urease, it does contain glutaminase and a variety of deaminating and transaminating enzymes acting upon amino-acids (Krebs, 1935*a*, *b*; Blanchard, Green, Nocito & Ratner, 1944; Mylon & Heller, 1948).

Support for this is found from two types of experiments with dogs *in vivo*. In the first, possible precursors of urinary ammonia were administered intravenously, and the effect on ammonia excretion determined by estimation of ammonia either in the urine or in the renal artery and vein. By such experiments, Polonovski, Boulanger & Bizard (1934), Polonovski & Boulanger (1938), Bliss (1941) and Lotspeich & Pitts (1947) suggest that glycine, D- or L-alanine, D- or L-leucine and DL-aspartic acid may act as precursors of urinary ammonia, whereas L-arginine, L-lysine and L-glutamic acid apparently do not. Such results are not conclusive evidence of the normal mechanism in the living organism. This is obvious in regard to the unnatural (D) forms of amino-acids which are certainly not the normal precursors, though their administration increases the excretion of urinary ammonia. It may also be true of the natural acids, since they do not normally occur in the high concentrations which result from the intravenous injections used in the experiments.

In the second type of *in vivo* experiment, estimations were made of the rate of removal from the blood of the possible precursors themselves, already present in the blood. In this way, Van Slyke *et al.* (1943) showed that L-glutamine was removed from the renal blood in amounts which account for 60%

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or more of the ammonia excreted. The rest of the ammonia was accounted for by a decrease in α-amino-acid nitrogen in the renal blood.

Our interest in adaptive enzymes suggested to us another approach to this problem (Yudkin, 1952). Since acidosis results in more urinary ammonia production and alkalosis results in less, it is possible that, in time, these conditions might produce an increase or a decrease in some of the renal enzymes concerned in ammonia production. The possibility that such an adaptive response occurs is supported by the fact that the increase in urinary ammonia induced by chronic acidosis reaches its maximum only gradually. The experiments to be described show that adaptation does in fact occur; of five renal enzymes examined, three showed an appreciable increase in chronic acidosis and an appreciable decrease in chronic alkalosis.

EXPERIMENTAL

Enzyme activity was measured in kidney slices from normal rats and from rats rendered chronically acidotic and alkalotic.

Animals and diets. Albino rats of the Wistar strain were used throughout. Litter mates of the same sex were distributed into three groups of six animals. Each group was housed in one cage and fed and watered *ad lib.* One group received water only, one received 0·05 N-HCl and the third 0·1 N-$NaHCO_3$ (see below). Apart from the experiments with L-glutaminase, the rats were fed from weaning on a stock diet consisting of cubes, with supplements of milk and greens (see Wiesner & Yudkin, 1951). The experiment with L-glutaminase was the first of these series and the rats were given diet AX, consisting of national bread 30%, sucrose 35%, light white casein 20%, dried baker's yeast 5%, arachis oil 3%, cod-liver oil 0·5% and salt mixture 6·5%. The animals received this from the age of 12 weeks, together with water, acid or alkali. By the time this experiment was completed, it had been established that growth on this diet was slightly, though not significantly, inferior to that on the laboratory stock diet; in all other experiments therefore the stock diet was used.

The animals were maintained on water, acid or alkali for at least 3 months, to ensure adequate time for adaptation to occur. For practical reasons, it was not possible to estimate enzyme activity in each experiment after equal times, and these were carried out from 3 to 8 months from the beginning of the experiment. Again, for practical reasons, male animals were used in some experiments and female animals in others. Table 4 summarizes some of the conditions in the experiments performed. Although these different conditions might have affected the absolute values found, they do not affect the conclusions drawn in each experiment from comparison of the groups receiving water, acid or alkali.

Induction of acidosis and alkalosis. The method chosen for induction of acidosis and alkalosis was the substitution of dilute solutions of HCl or of $NaHCO_3$ for the drinking water, to which the animals had constant access. A strength of 0·1 N-HCl proved unpalatable so that this was reduced to 0·05 N; $NaHCO_3$ was given as 0·1 N. That this method produced substantial changes in the production of urinary ammonia was determined as follows. After 3 months on this regime, six rats given water, acid or alkali were transferred to individual metabolism cages, the urine collected for 24 hr. and ammonia estimated by the aeration method of Van Slyke (see Hawk, Oser & Summerson, 1947). Since it was desired to ascertain only the existence of obvious differences in ammonia production, no washings of the cages and funnels were made for addition to urine. Significant differences occurred in urinary ammonia in both male and female animals (Table 1). It is interesting to observe that appreciable differences exist in ammonia excretion in male and in female animals. This may be due to the differences in weight; the average weight of the males was 285 g. and of the females 175 g.

Table 1. *Excretion of urinary ammonia in normal, acidotic and alkalotic animals*

(Six animals in each group, fed stock diet and given water, acid or alkali for 3 months.)

Sex of animals	Urinary ammonia produced in 24 hr. (mean mg. NH_3-N ± S.E.)		
	Normal	Acid	Alkali
Male	11·1 ± 0·86	21·6 ± 0·77	2·3 ± 0·10
Female	8·7 ± 0·13	12·1 ± 0·48	2·0 ± 0·13

Table 2 shows that the excretion of ammonia varied little from day to day. As a routine in later experiments therefore, one rat from each group was tested a day or two before the estimation of enzyme activity, to ensure the persistence of acidosis or alkalosis in the group.

Table 2. *Constancy of urinary ammonia*

(One male rat from each group, fed stock diet and given water, acid or alkali for 3 months; urinary ammonia measured for 3 consecutive days.)

Group	Urinary ammonia in 24 hr. (mg. NH_3-N)		
	Day 1	Day 2	Day 3
Water	12·4	12·1	12·3
Acid	24·4	23·9	24·1
Alkali	2·9	3·0	2·9

Enzymes. Ammonia may arise from amino-acids by deamidation, oxidative deamination or transamination. In order to examine possible adaptation in all these processes, the enzymic production of ammonia from the following five substances was studied: L-glutamine, glycine, L-leucine, L-aspartic acid and L-alanine. The pertinent data determining the selection may be summarized as follows: Van Slyke *et al.* (1943) claimed that L-glutamine is the chief source of urinary ammonia; their evidence suggests that 60% or more is derived from this substance. Glycine is one of the amino-acids which increase ammonia excretion when administered to acidotic dogs (Lotspeich & Pitts, 1947). Glycine oxidase is specific for this substance (Ratner, Nocito & Green, 1944). The experiments of Lotspeich & Pitts (1947) suggest that L-leucine, a monoamino monocarboxylic acid, may also be a precursor of urinary ammonia. It is deaminated by L-amino-acid oxidase and its deamination *in vitro* is at a greater rate than any other monoamino

monocarboxylic acid (Blanchard *et al.* 1944). Lotspeich & Pitts (1947) showed that DL-aspartic acid increased urinary ammonia in acidotic dogs. The rate of deamination *in vitro* is greater than that of any other amino-acid (Krebs, 1935*a*). L-Aspartic acid is not affected by L-amino-acid oxidase (Blanchard *et al.* 1944) but appears to be deaminated by a transaminase system (Braunstein, 1947).

The four preceding enzymes cover the processes of deamidation, oxidative deamination of glycine, oxidative deamination of other naturally occurring (L) amino-acids, and transamination. Nevertheless, it was thought worth while also to examine the deamination of L-alanine, since this may be effected both by L-amino-acid oxidase (Blanchard *et al.* 1944) and by transamination (Braunstein, 1947; Krebs, 1948).

Enzyme assay. The selected rat was killed by a blow on the head and the kidney rapidly removed, decapsulated and weighed. A portion was removed, weighed and again weighed after drying at 110°. Slices of 0·3 mm. were cut by the method of Deutsch (1936) from material kept ice-cold and moistened with oxygenated Ringer-phosphate solution of pH 7·4. Ammonia production was measured in the presence of O_2 in Warburg manometers at 38°. The flasks contained 2·5 ml. Ringer-phosphate solution and the side arm 0·5 ml. neutralized substrate solution. Ammonia production from kidney slices in the absence of substrate was examined at the same time in flasks containing 3·0 ml. Ringer-phosphate and these control values were subtracted from the values obtained in the presence of substrate. At the end of the experiment, the tissue slices were removed, dried and weighed. The ammonia produced was measured either by Van Slyke's aeration method or by the HCl-$Ba(OH)_2$ method of Conway (Conway, 1947). Enzyme activity was expressed as μg. NH_3-N produced from the substrate/hr. in these conditions by selected dry weights of kidney tissue and by the whole kidney. Control values are reported for all experiments except for those with L-glutamine, where the blanks were less than 10% of the effect. With each enzyme, preliminary experiments were carried out to ascertain optimal substrate concentration, time of incubation and appropriate amounts of tissue to be used.

Glutamine. It was found that slices of cortex had the same enzyme activity as slices of cortex with medulla, so that the experiments with this enzyme were carried out with slices including both. From initial experiments, it was decided to use 4 mg. L-glutamine (final concentration 0·01 M approx.) and an incubation time of 30 min.; this gave reasonable yields of ammonia which, even in the most active tissues, were appreciably below the theoretical yield of 381 μg. As the relation between ammonia produced and tissue used was not quite linear, four different amounts of tissue, with dry weights assessed at between 5 and 14 mg., were used for each rat, and an enzyme activity curve constructed. From this, enzyme activity was calculated by interpolation for dry weights of tissues of 6, 8, 10 and 12 mg.

Other substrates. Slices of renal cortex were used with these substrates. Trial experiments led to the adoption of the following final concentrations of substrate and times of incubation: glycine, 0·01 M and 90 min.; L-leucine, 0·01 M and 60 min.; L-aspartic acid, 0·005 M and 60 min.; L-alanine, 0·01 M and 120 min. These conditions were determined partly by the solubility of the substrate and partly by the need to have maximal production of ammonia without exhaustion of substrate or sensible deterioration of enzyme activity.

For these four substrates, calibration curves were constructed relating enzyme activity and amount of tissue. Each curve was linear up to about 15 mg. dry weight of tissue. In the estimation of enzyme activity, therefore, kidney slices were rapidly weighed before placing in the manometers and amounts used which would give dry weights between 10 and 15 mg. For each animal, these experiments and their controls were carried out in duplicate.

RESULTS

Growth of animals

In each of the five experiments, there were slight differences in weight in the normal, acidotic and alkalotic groups (Table 3). The differences between the normal and alkalotic animals were slight and variable; the differences between either of these and the acidotic animals were greater and consistently in favour of the normal and alkalotic groups. Although the differences were statistically significant in Exp. 2 only, it seems that chronic acidosis over a long period may produce slight impairment of growth.

Table 3. *Effect of chronic acidosis and chronic alkalosis on growth of rats*

(Six animals in each group. Diets and duration of experiment as in text. Mean wt. ± S.E.)

Exp. no.	Substrate investigated	Wt. of animals at end of adaptation (g.) Normal	Acid	Alkali
1	L-Glutamine	194 ± 4·1	193 ± 1·1	194 ± 3·4
2	Glycine	361 ± 4·4	337 ± 3·2	368 ± 6·4
3	L-Leucine	215 ± 5·8	204 ± 2·7	217 ± 6·2
4	L-Aspartic acid	352 ± 4·4	330 ± 11·6	342 ± 8·5
5	L-Alanine	346 ± 12·2	340 ± 10·5	347 ± 8·7

(Differences between 'normal' and 'acid' groups and 'acid' and 'alkali' groups in Exp. 2 are significant ($P = 0·01$). Differences in other experiments are not significant ($P > 0·05$).)

Enzyme activity

Glutamine. Acidosis resulted in a significant increase in glutaminase and alkalosis in a significant decrease (Table 5).

Other substrates. Since these experiments were made with renal cortex only, it is not possible to calculate the absolute amounts of enzyme in the whole kidney. If, however, it is assumed that the relative proportions of cortex and medulla were the same in all the groups, it is possible to calculate the relative amounts of total enzyme from the measured activity, the weight of the tissue used and the weight of the kidney. We have therefore given the arbitrary figure of 100 as the total activity of the kidneys from normal animals; the activities of acidotic and alkalotic animals are given as a proportion of this.

Table 4. *Summary of some of the experimental procedures*

Exp. no.	Substrate investigated	Sex of animals	Age of animals (weeks): Beginning of adaptation	Age of animals (weeks): End of adaptation	Duration of adaptation (weeks)	Concentration of substrate (M)	Time of incubation (min.)
1	L-Glutamine	Female	13	26	13	0·01	30
2	Glycine	Male	3	33	30	0·01	90
3	L-Leucine	Female	3	21	18	0·01	60
4	L-Aspartic acid	Male	3	24	21	0·005	60
5	L-Alanine	Male	3	33	30	0·01	120

Acidosis resulted in a significant increase, and alkalosis in a significant decrease, in the enzymes producing ammonia from glycine and from L-leucine (Table 6). When calculated on the basis of unit weight of kidney, acidosis resulted in a slight though significant increase in the enzyme producing ammonia from L-aspartic acid. This small change is probably fortuitous, since it disappeared when the total activity of the kidney was calculated. It seems legitimate to conclude that, as far as the whole kidneys are concerned, neither acidosis nor alkalosis resulted in a significant change in the enzymes producing ammonia from L-aspartic acid or from L-alanine.

Table 5. *Effect of chronic acidosis and chronic alkalosis on renal ammonia production from* L-*glutamine*

(Slices incubated for 30 min. with 0·01 M-L-glutamine. Kidney wt.: differences between any two groups are not significant ($P > 0·05$). Enzyme activity: in sections (*a*) and (*b*) differences between any two groups are significant ($P < 0·01$).)

(*a*) Activity/unit weight of kidney cortex. Values, corrected for blanks, were calculated by interpolation from experimental values and are expressed as μg. NH_3-N/mg. dry wt. of tissue/hr. ± S.E. for six animals in each group.

Dry wt. of tissue (mg.)	Group: Normal	Acid	Alkali
6	22±0·9	42±5·1	14±1·4
8	22±0·8	40±4·4	16±1·3
10	19±0·6	36±3·3	15±0·8
12	17±0·6	33±2·6	13±0·8

(*b*) Activity for whole kidney. Calculated for intrapolated values for 10 mg. dry wt.

Group	Dry wt. of total kidney (mg.)	Ammonia produced ± S.E.: (μg. NH_3-N/mg. dry wt./hr.)	Ammonia produced ± S.E.: (μg. NH_3-N/total kidney/hr.)
Normal	380±18	19±0·6	7 280±450
Acid	370± 7	36±3·3	13 400±1 330
Alkali	380±10	15±0·8	5 820±380

Thus, increase or decrease in ammonia production induced by acidosis or alkalosis results in an adaptive response in three of the five enzyme systems studied—those concerned in ammonia production from L-glutamine, glycine and L-leucine.

DISCUSSION

This work on enzyme adaptation provides evidence that the sources of urinary ammonia include L-glutamine, glycine and L-leucine. This is in accord with the results referred to earlier, in which it was shown that the intravenous administration of these substances increase the amount of ammonia excreted.

The present work provides no new evidence concerning the participation of L-alanine or L-aspartic acid in ammonia production. No adaptive changes were found, but, as has been pointed out elsewhere (Yudkin, 1952), not all enzymes are capable of showing adaptation. It would appear from this, therefore, that the enzymes concerned in the deamination of these two amino-acids either are not concerned in ammonia production or are not adaptive enzymes.

This question is not answered by the results of other workers. We have seen that Bliss (1941) and Lotspeich & Pitts (1947) found that the administration of D- or L-alanine or of DL-aspartic acid increased urinary ammonia. The latter authors have themselves questioned whether ammonia could be formed from the L-isomer in the experiments with DL-aspartic acid, since the injection of another L-monoamino dicarboxylic acid, L-glutamic acid, failed to augment urinary ammonia. In addition, as we have pointed out, the concentrations of the amino-acids following injection are abnormally high. It remains therefore that no decision can be reached at this stage concerning the participation or otherwise of L-alanine and L-aspartic acid in renal ammonia production.

It is interesting to examine more closely the enzymes involved in ammonia production from L-alanine and L-aspartic acid. Both may be acted upon by the transaminase system, whilst L-alanine may also be acted upon by L-amino-acid oxidase.

Our results with L-leucine have shown that acidosis or alkalosis leads to a considerable increase or decrease in L-amino-acid oxidase. Since, however, these conditions do not affect ammonia production from L-alanine, it must be concluded that this amino-acid is acted upon very little, if at all, by the L-amino-acid oxidase of kidney slices. In this connexion, it is relevant to recall that alanine is the least rapidly deaminated amino-acid of the 13 acted upon by purified L-amino-acid oxidase; the rate is, for example, only 3% of that of L-leucine (Blanchard *et al.* 1944).

enzyme adaptation support the participation of the first three of these but can provide no evidence for or against the participation of the last.

Whilst the work described here was in progress, Handler, Bernheim & Bernheim (1949) reported experiments along similar lines. They found no change in ammonia production from L-alanine, L-glutamine or L-leucine by kidney slices of normal rats compared with those of rats rendered acidotic or alkalotic. Though their results with L-alanine agree with ours, their results with L-leucine and L-glutamine do not. However, examination of their paper

Table 6. *Effect on chronic acidosis and chronic alkalosis on renal ammonia production from glycine, L-leucine, L-aspartic acid and L-alanine*

(Mean values, corrected for blanks for six animals in each group ± S.E. Figures in brackets are mean control values. Measurements with different substrates were made under different conditions, as given in Table 4. Comparisons may therefore be made horizontally, but not vertically.)

	Ammonia produced (μg. NH_3-N/mg. dry wt./hr.)			Dry wt. of total kidney (mg.)			Ammonia produced by total kidney (Arbitrary units; ammonia produced by normal rats = 100)		
Substrate	Normal	Acid	Alkali	Normal	Acid	Alkali	Normal	Acid	Alkali
Glycine	0·64 ± 0·01 (1·05)	1·59 ± 0·07 (1·09)	0·43 ± 0·01 (0·99)	450 ± 9	430 ± 17	430 ± 11	100 ± 2·5	236 ± 14·1	63 ± 2·1
L-Leucine	1·94 ± 0·05 (1·15)	3·01 ± 0·03 (1·14)	1·02 ± 0·03 (1·16)	330 ± 11	340 ± 10	340 ± 21	100 ± 1·9	157 ± 5·2	53 ± 4·2
L-Aspartic acid	3·53 ± 0·05 (1·16)	3·81 ± 0·06 (1·13)	3·73 ± 0·11 (1·15)	520 ± 25	470 ± 14	460 ± 7	100 ± 4·7	98 ± 3·5	94 ± 3·4
L-Alanine	0·28 ± 0·01 (1·01)	0·28 ± 0·01 (1·08)	0·28 ± 0·01 (1·02)	480 ± 24	490 ± 30	460 ± 16	100 ± 4·4	101 ± 6·0	93 ± 4·5

Kidney wt.: difference between any two groups not significant ($P > 0·05$), except difference between 'normal' and 'alkali' groups with L-aspartic acid ($P = 0·04$). Enzyme activity: with glycine and L-leucine, differences between any two groups significant ($P < 0·01$); with L-aspartic acid and L-alanine, differences between groups not significant ($P > 0·05$), except for difference between 'normal' and 'acid' groups with L-aspartic acid, calculated on basis of unit wt. of kidney ($P = 0·01$).

It seems then that L-alanine is deaminated chiefly, if not entirely, by the transaminase system and our results therefore suggest that chronic acidosis or alkalosis produce no change in this system. This may be because it plays no part in the formation of urinary ammonia. It might, on the other hand, be that the transaminase system is not adaptive; L-alanine and L-aspartic acid may in fact be precursors of urinary ammonia, but adaptation of this system does not occur. Since the transaminase system consists of more than one enzyme (probably a dehydrogenase and a transaminase), it is possible to imagine that one of them does in fact respond adaptively but that the other does not, and limits the rate of ammonia production. The effect would be the same in that the system as a whole would not appear to be adaptive.

This discussion may now be summarized in the following way. Experiments *in vivo* suggest that urinary ammonia may be produced by four enzyme systems: L-glutaminase, glycine oxidase, L-amino-acid oxidase and transaminase. The experiments on

makes it difficult to accept their findings without criticism. With L-leucine, they used only one normal and one acidotic animal. In addition, the rate of deamination reported with this substrate was lower than with any other, which is not in conformity with the results of other workers. With L-glutamine, they used only one normal, one acidotic and one alkalotic animal. More important, however, the conditions under which they measured ammonia production were such that about 90% of the possible maximal amount of ammonia was produced from the glutamine. It is clear that, in these circumstances, it might prove quite impossible to detect differences in enzyme activities.

Our experiments also provide evidence in another matter which has been the subject of discussion. This is whether an increase in urinary ammonia is caused by an increase in actual production of ammonia or, as suggested by Krebs (1935*a*) and Polonovski, Boulanger & Bizard (1935), an increase merely in the amount removed from the blood. The fact that chronic acidosis is accompanied by an

increase in enzymes responsible for ammonia production favours the view that increased urinary ammonia is caused, at least in part, by an increased formation of ammonia.

SUMMARY

1. Changes in ammonia excretion were produced in rats by the induction of chronic acidosis and chronic alkalosis. After 3–8 months, some selected renal enzymes were assayed in these animals by determining ammonia production, by kidney slices, from L-glutamine, glycine, L-leucine, L-aspartic acid and L-alanine. These substrates were selected to investigate possible adaptation of four enzymes which might be concerned in ammonia production: L-glutaminase, glycine oxidase, L-amino-acid oxidase and the transaminase system.

2. Chronic acidosis resulted in an appreciable increase, and chronic alkalosis in an appreciable decrease, in ammonia production from L-glutamine, glycine and L-leucine. There was no significant change in ammonia production from L-aspartic acid and L-alanine.

3. These results suggest that urinary ammonia is produced, at least in part, by (1) deamination of glutamine, (2) deamination of glycine by glycine oxidase, (3) deamination of other monoamino monocarboxylic acids by L-amino-acid oxidase.

4. Reasons are given for supposing that deamination of L-alanine is effected chiefly, if not entirely, by the transaminase system which also acts upon L-aspartic acid. It would then appear either that this system plays no part in the normal mechanism of urinary ammonia formation, or that one or more of its component enzymes is not adaptive.

5. The adaptive changes found in three renal enzymes indicate that acidosis and alkalosis cause a change in ammonia formation and not merely in ammonia elimination.

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Kinetic Studies of the Metabolism of Foreign Organic Compounds

2. THE FORMATION OF PHENOLS FROM CERTAIN PRECURSORS

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The introduction of a hydroxyl group into the benzene nucleus is recognized as a common metabolic process. Compounds which are hydroxylated in this way may be regarded as phenol precursors. The present investigation deals with phenol precursors which are hydroxylated to give predominantly one isomer, namely anisole, which gives *p*-methoxyphenol (Bray, Thorpe & Wasdell, 1951*a*), *N*-phenylurea, which gives *N*-(*p*-hydroxyphenyl)-urea (Bray, Lake & Thorpe, 1949) and benzoxazolone, which is excreted as a hydroxybenzoxazolone, the configuration of which has not yet been established (Bray, Clowes & Thorpe, 1952*a*). The overall plan was as described previously (Bray, Thorpe & White, 1951*b*). In some experiments the metabolites were estimated as phenols, in the

remainder as ethereal sulphate and glucuronide. The results obtained suggest that the rate of formation of phenols from these compounds is proportional to the body level of the precursor.

METHODS

Animals, diet, dosage and collection of urine samples

The animals were maintained on the standard pellet diet previously described (Bray, Ryman & Thorpe, 1947). The compounds (usually 0·7 g.) were administered by stomach tube as suspensions in water, either alone or, more often, with Na_2SO_3 or L-cystine. Administration of these 'sulphate precursors' is usually necessary to ensure that the ethereal sulphate conjugation follows first-order reaction kinetics. This is discussed in detail in a subsequent paper (Bray, Humphris, Thorpe, White & Wood, 1952*c*). The collection of urine samples was as previously described (Bray *et al.* 1951*b*). The water intake was considerable.

Estimation of metabolites

Free and total phenols. A modification of the Folin & Ciocalteu method described by Bray, Thorpe & White (1950*b*), was used with a Spekker photoelectric absorptiometer with Chance filter OR 2. The quantities suitable for estimation varied with the phenol but were of the order of 0·1 mg. The slope of the linear calibration curve was of the order of 0·025 mg./0·1 drum reading.

Glucuronic acid. The method was a modification of that described by Hanson, Mills & Williams (1944), the chief alteration being the replacement of amyl alcohol by *n*-butanol, thus obtaining a monophasic system so that separation before reading is unnecessary. This greatly facilitates the analysis of a large number of urine samples.

Reagents. Naphthoresorcinol solution, 0·375% (w/v) in water, was 'aged' by keeping either at room temperature for 2 days or at 0° for 1 week before use. The 'aged' solution can be kept at 0° for 1 week.

Undiluted (approx. 10 N) technical grade hydrochloric acid (British Drug Houses Ltd.) was used. The blue colour may not develop if pure HCl is used. The *n*-butanol was British Drug Houses Ltd. ordinary quality. The D-glucurone solution contained 0·1 mg./ml. in water and if kept at 0° was stable for 1 week.

Procedure. The urine was diluted so as to contain the equivalent of not more than 0·04 mg./ml. glucuronic acid. Diluted urine (2 ml.) was placed in a glass tube (13 × 150 mm.), and HCl (4 ml.) and naphthoresorcinol solution (2 ml.) added. The tube was heated in boiling water for 2 hr., evaporation being minimized by the use of a 'cold finger', and then cooled in cold water. After addition of *n*-butanol (5 ml.) the tube was corked, shaken and then left for 2–3 min. to disperse air bubbles. The mixture did not form two layers, thus eliminating the separation of the coloured organic phase (Hanson *et al.* 1944). The absorption value was read in a Spekker photoelectric absorptiometer within 5 min. of the addition of the butanol. An Ilford Spectrum Orange filter 607 was used. If absorption values were read 30 min. after mixing with butanol results were from 0·001 to 0·003 mg. (3–5%) lower per tube. A calibration curve was constructed using, in place of urine, from 0·2 to 2·0 ml. glucurone solution made up to 2 ml. with water. D-Glucuronic acid, D-glucurone and 3:4-dimethylphenylglucuronide in water or urine have been found to give identical linear calibration curves (drum reading 0·1 ≡ 0·015 mg. glucuronic acid per tube). If the naphthoresorcinol solution is insufficiently 'aged' the calibration curve is not linear above 0·05–0·10 mg. glucuronic acid.

Inorganic and ethereal sulphate. A simplified turbidimetric method was used. The following reagents were required: hydrochloric acid, about 7·1 N; sodium hydroxide-potassium sulphate solution, NaOH (2·5 N) containing K_2SO_4 (0·128 g./l.) (the sulphate is added to ensure that the absorption calibration curve is linear over the required range); $BaCl_2.2H_2O$, 'Analar'; potassium sulphate solution, K_2SO_4 0·44 g./l.

Procedure. Estimations of inorganic and total sulphate were carried out simultaneously so as to ensure uniformity in conditions. For total sulphate, urine (5 ml. containing 0·1–1·0 mg. SO_3) was heated with HCl (2 ml.) in a tube (16 × 150 mm.) in boiling water for 1 hr., evaporation being reduced by use of a 'cold finger'. To this solution, after cooling, NaOH-K_2SO_4 (5 ml.) was added. For inorganic sulphate, NaOH-K_2SO_4 (5 ml.) and HCl (2 ml.) were mixed in a test tube and, after cooling, urine (5 ml.) was added. The tubes for both inorganic and total sulphate were prepared in duplicate. To one of each $BaCl_2.2H_2O$ (200 mg.) was added and the tube vigorously shaken for 30 sec. The absorption value of each of the four solutions was determined with a Spekker photoelectric absorptiometer using a Chance neutral filter H 508. Absorption values were read between 7 and 10 min. after mixing. The $BaSO_4$ suspension was stable up to 20 min. A calibration curve was made by using appropriate dilutions of the K_2SO_4 solution. The calibration curve was linear over the range 0–1·0 mg. SO_3 per tube and identical curves were given by potassium, ammonium and ferrous ammonium sulphates. For estimation of ethereal sulphate in urine this method gave results averaging 92% (range 80–105%) of those obtained by the Folin (1905–6) gravimetric method. The average recovery of inorganic sulphate added to urine was 94% (range 82–110%). The presence of sulphite did not interfere with the determination of inorganic or ethereal sulphate by the turbidimetric method. The method was reliable if the ratio inorganic/ethereal sulphate did not exceed 5·0.

THEORETICAL

Expressions relating the amounts of a phenol precursor administered and the amounts of the corresponding phenol (free and conjugated) formed in a given time after administration of the precursor can be derived as follows:

Assuming that an amount A of the precursor is introduced into the body at zero time and that it is hydroxylated by a process having a first-order velocity constant k_b, the resulting phenol being conjugated by processes having a total first-order velocity constant k_m, then the following differential equations may be formulated:

$$\frac{db}{dt} = -\frac{da}{dt} = k_b a,$$

$$\frac{dm}{dt} = k_m (b - m),$$

where a = amount of precursor at time t, b = total amount of phenol (free and conjugated) at time t, and m = amount of conjugated phenol at time t.

Solution of these equations gives

$$a = Ae^{-k_b t}, \quad (1)$$

$$b - m = \frac{k_b A}{k_m - k_b}(e^{-k_b t} - e^{-k_m t}), \quad (2)$$

$$m = A\left[1 - \left(\frac{k_m e^{-k_b t} - k_b e^{-k_m t}}{k_m - k_b}\right)\right]. \quad (3)$$

The kinetic studies reported in this and succeeding papers suggest that the above equations represent the course of the metabolic reactions which occur when a phenol precursor is administered to the rabbit. It will be shown (Bray *et al.* 1952*b*, *c*) that under the conditions of the present experiments the conjugation processes of phenols with glucuronic and sulphuric acids do, in fact, follow first-order reaction kinetics (with velocity constants k_g and k_s respectively), and that excretion of these conjugates by the kidney is efficient, so that their blood level remains low, and their rate of excretion may be taken as being equal to their rate of formation. In general, the rate of formation of the phenol may be found by applying the above equations to experimental data, namely the value of m at different times. Theoretical calculations show, however, that if the value of k_m is at least five times as great as that of k_b, then the rate of excretion of the conjugates will represent, to a close approximation, the rate of formation of the phenol, so that an excretion curve (cf. Bray *et al.* 1951*b*) may be taken as representing the formation of the phenol and graphical treatment applied to deduce a value for k_b.

RESULTS

Anisole. Values of k_b for anisole were calculated by substitution in equation 3 of the mean value for k_m (1·08 hr.$^{-1}$) found for *p*-methoxyphenol (obtained from $k_g + k_s$, Bray *et al.* 1952*b*, *c*) and of the observed values of t and m for anisole. The necessary assumption involved, namely, that the hydroxylation process follows first-order reaction kinetics, was vindicated by the fact that consistent values of k_b were obtained from several different points in any single experiment. The mean values of k_b found in two experiments were 0·20 and 0·16 hr.$^{-1}$. Since these values are less than one-fifth of that of k_m (1·08 hr.$^{-1}$), graphical methods (usually the 'log' method) were then applied (see Bray *et al.* 1951*b*) to all the anisole experiments. It was shown, by the fit of experimentally determined points on theoretical curves corresponding to first-order velocity constants derived by the graphical methods, that the hydroxylation of anisole follows first-order reaction kinetics, i.e. the rate of the reaction is proportional to the amount of anisole in the body, designated 'body level'. The results for a typical experiment are shown in Fig. 1. The values obtained for k_b are given in Table 1. It should be noted that in the derivation of equation 3 it is assumed that the precursor is introduced into the body at zero time. Since experimentally the compounds are absorbed from the alimentary tract, zero time is not known exactly, but extrapolation of the excretion curve to meet the time axis at t_o, as shown for the theoretical curve in Fig. 1, gave values for zero time leading to consistent values for k_b provided that only experimental points after 2–3 hr. were used for the calculation.

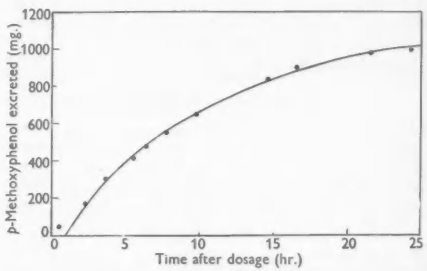

Fig. 1. Excretion of *p*-methoxyphenol by the rabbit after administration of 1 g. anisole. The curve is the theoretical curve for $k_b = 0{\cdot}10$ hr.$^{-1}$ and the points are those obtained by experiment.

Table 1. *Velocity constants (in hr.$^{-1}$) for the hydroxylation of anisole, N-phenylurea and benzoxazolone in the rabbit, determined graphically using the 'log' method*

(Dose 0·7 g. unless stated otherwise.)

Anisole	*N*-Phenylurea	Benzoxazolone
0·10*	0·12	0·21
0·14	0·15	0·37
0·17	0·10	0·21
0·06	—	0·21
—	—	0·29
0·11†	0·12†	0·25†

* Dose 1·0 g.
† Geometric mean, see Bray *et al.* (1952*b*), Table 2.

N-Phenylurea and benzoxazolone. By similar application of equation 3 to data from two experiments with *N*-phenylurea and two with benzoxazolone (for values for k_g and k_s see Bray *et al.* 1952*b*, *c*) the mean values for k_b obtained were 0·20 and 0·21 hr.$^{-1}$ for *N*-phenylurea and 0·35 and 0·37 hr.$^{-1}$ for benzoxazolone. Since the mean values of k_m for the phenols concerned are respectively 0·22 and 0·36 hr.$^{-1}$, the values for k_b derived graphically cannot be taken as true values without further consideration. Values of k_b derived by the

'log' method from excretion curves representing the same experimental data were 0·10 and 0·15 hr.$^{-1}$ for *N*-phenylurea and 0·29 and 0·37 hr.$^{-1}$ for benzoxazolone, i.e. a mean of 0·6 and 0·9 of the mathematically derived values respectively. Since calculation of the mean value of k_b by means of equation 3 involves the solution of the equation by successive approximation for each experimental observation, it is impracticable to treat any considerable number of experiments in this way. The results reported in Table 1 are those derived by the 'log' method as for anisole. It is more likely, however, that the correct mean values of k_b for *N*-phenylurea and benzoxazolone would be $0{\cdot}12/0{\cdot}6 = 0{\cdot}20$ and $0{\cdot}25/0{\cdot}9 = 0{\cdot}28$ hr.$^{-1}$ respectively. The present investigation was primarily intended to supply results necessary for the investigations reported in the next three papers. For this purpose this degree of accuracy is sufficient.

DISCUSSION

Although the values of k_b for the three compounds discussed here are within the range 0·1–0·3 hr.$^{-1}$, it is probable that in general there is a wide range of values for k_b. For example, with compounds which form only traces of hydroxylation products, e.g. *p*-cresol (Bray *et al.* 1950*b*), the value is probably of the order of 0·001–0·01 hr.$^{-1}$. It is possible to calculate an approximate value for k_b for *p*-hydroxybenzamide. This compound, which is hydrolysed to the extent of 10%, has a first-order velocity constant for the hydrolysis of the carbamyl group of 0·05 hr.$^{-1}$ (unpublished result). The extent of hydroxylation is 1–4% of the dose (Bray, Thorpe & White, 1950*a*). Thus k_b for this compound must be about 0·005–0·02 hr.$^{-1}$.

This series of investigations had as its primary stimulus an implication of the generalization (Thorpe, 1950) relating to the metabolism of foreign organic compounds which stated that conjugation appeared to take place more readily than conversion of potential centres for conjugation, and conversion of potential centres more readily than hydroxylation. The implication was that this was a consequence of the values of the velocity constants of the three types of reaction. It was thus expected that the velocity constants of conjugation reactions would be appreciably greater than those of processes for the conversion of potential centres and these, in their turn, greater than the velocity constants of hydroxylation reactions. It can be seen, however, from the examples given in Table 2 that certain values of velocity constants for hydroxylation are comparable in magnitude with those found for conjugation reactions and conversion processes, and that the velocity constants of conjugation processes are not always greater than those of hydroxylation processes.

Table 2. *Mean velocity constants (in hr.$^{-1}$) for conjugation, conversion and hydroxylation reactions in the rabbit*

Conjugations:	
Benzoic acid with glucuronic acid*	0·08
N-*p*-Hydroxyphenylurea with glucuronic acid†	0·11
N-*p*-Hydroxyphenylurea with sulphuric acid‡	0·11
Hydroxybenzoxazolone with glucuronic acid†	0·18
Hydroxybenzoxazolone with sulphuric acid‡	0·18
p-Methoxyphenol with glucuronic acid†	0·54
p-Methoxyphenol with sulphuric acid‡	0·54
Conversions of potential centre for conjugation:	
Toluene to benzoic acid*	0·11
Benzamide to benzoic acid*	0·32
Hydroxylations:	
N-Phenylurea to *N*-*p*-hydroxyphenylurea§	0·20
Benzoxazolone to hydroxybenzoxazolone§	0·28
Anisole to *p*-methoxyphenol§	0·11

* Bray *et al.* (1951*b*). † Bray *et al.* (1952*b*).
‡ Bray *et al.* (1952*c*). § This paper.

SUMMARY

1. An investigation of the kinetics of hydroxylation of anisole, *N*-phenylurea and benzoxazolone in the rabbit has been made.

2. The rates of hydroxylation of these compounds appear to be proportional to their body levels, the velocity constants being 0·11 hr.$^{-1}$ for anisole and approximately 0·20 and 0·28 hr.$^{-1}$ for *N*-phenylurea and benzoxazolone respectively.

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Kinetic Studies of the Metabolism of Foreign Organic Compounds

3. THE CONJUGATION OF PHENOLS WITH GLUCURONIC ACID

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It is well known that phenols, whether formed *in vivo* or administered as such, are excreted by many species largely conjugated with glucuronic and sulphuric acids. The extent of the conjugation with glucuronic acid has been examined for many phenols and by many workers, the result usually being recorded as a percentage of the dose excreted as glucuronide in 24 or 48 hr. Several of the glucuronides have been isolated from urine in pure form. Studies of the formation and hydrolysis of phenylglucuronides by tissue extracts or slices have also been made, but the mechanism of glucuronide formation *in vivo* is as yet obscure. The present paper records the results of an investigation of the kinetics of glucuronide formation. This aspect does not appear to have been investigated previously.

METHODS

Animals, diet and collection of urine specimens. The rabbits, does of average weight 2·8 kg., were maintained on the standard diet of rabbit pellets used previously (Bray, Ryman & Thorpe, 1947). The apparatus for the collection of urine specimens has been described (Bray, Thorpe & White, 1951).

Dosage. The compounds were administered by stomach tube as solutions or suspensions in water, at dose levels usually between 0·15 and 0·45 g./kg. Since many of the experiments described here also formed part of the study of the kinetics of ethereal sulphate formation described in the next paper, Na_2SO_3 or L-cystine was often administered with the phenol. This did not appear to affect the kinetics of glucuronide formation.

Materials. *p*-Cyanophenol was prepared by a Sandmeyer reaction from *p*-aminophenol; *p*-hydroxybenzamide as described by Bray *et al.* (1947), *p*-hydroxybenzenesulphonamide by decomposition of the diazonium salt of *p*-aminobenzenesulphonamide; *p*-hydroxyphenylurea as described by Bray, Lake & Thorpe (1949). Hydroxybenzoxazolone was isolated from urines of rabbits dosed with benzoxazolone (Bray, Clowes & Thorpe, 1952*a*). Other phenols were purchased.

Estimation of metabolites

Methods used for the estimation of glucuronic acid, inorganic and ethereal sulphates and free phenols have been described in the previous paper (Bray, Humphris, Thorpe, White & Wood, 1952*b*). Estimation of glucuronides in blood was carried out on a trichloroacetic acid filtrate of whole blood. Xylenylglucuronides were also estimated by determination of the ether-soluble acid on a sodium tungstate filtrate of whole blood.

p-*Hydroxybenzoic acid.* The above method for phenols is unsuitable for the estimation of *p*-hydroxybenzoic acid which gives only a feeble colour with the Folin and Ciocalteu reagent. The method to be described is based upon the colour formed when *p*-hydroxybenzoic acid is coupled with diazotized *p*-nitraniline.

The following reagents were required: *p*-nitraniline solution (3 mg./l. in 0·8 N-HCl); sodium nitrite solution (5% w/v in water); ethanol (96%); diazotized *p*-nitraniline solution (*p*-nitraniline, 25 ml., and sodium nitrite, 1·5 ml., mixed immediately before use); sodium carbonate solution (Na_2CO_3, 5% w/v in water). Urine (20 ml.) acidified with H_2SO_4 (2 ml. 2 N) was continuously extracted with ether for 6 hr. After removal of ether, the extract was titrated with NaOH (0·02 N) using phenol red as indicator. This gave the total ether-soluble acid. The solution was diluted so that the concentration of *p*-hydroxybenzoic acid was between 0·2 and 2 mg./100 ml. This solution (5 ml.) was mixed with ethanol (5 ml.) and diazotized *p*-nitraniline (1 ml.) and left for 2 min. Sodium carbonate solution (3 ml.) was added and the solution diluted to 25 ml. with water. The absorption value was read immediately on a Spekker photoelectric absorptiometer using a blue filter (Chance OB1).

A calibration curve was constructed using solutions containing 0·01–0·10 mg. *p*-hydroxybenzoic acid/5 ml. Within this range the curve was linear, and, when allowance was made for the reagent blank, passed through the origin. The slope was approximately 0·01 mg. acid ≡ 0·1 drum reading. The average recovery of *p*-hydroxybenzoic acid added to normal urine was 102% (range 96–110%). The difference between the total ether-soluble acid and the *p*-hydroxybenzoic acid values may be taken as representing *p*-hydroxyhippuric acid since this compound gives no significant colour under the above conditions.

p-*Hydroxybenzamide.* The titrated ether-soluble acid solution prepared as for the estimation of *p*-hydroxybenzoic acid (above) was diluted to 100 ml. with water. The diluted solution (5 ml.) was mixed with H_2SO_4 (5 ml. 10 N) in a test tube (16 × 150 mm.) and heated in boiling water for 2 hr. using a 'cold finger' as condenser. The mixture was cooled, treated with NaOH (10 ml. 5 N), again cooled and diluted to 25 ml. The *p*-hydroxybenzoic acid in this solution (further diluted if necessary in order to obtain a concentration of 0·01–0·1 mg. *p*-hydroxybenzoic acid/5 ml.) was estimated as described above.

Plan of experiments

This was in general as described previously (Bray *et al.* 1951), except that since the excretion of administered compounds was complete within 6–16 hr. the ordinary food

ration could be withheld until the end of the experiment. Phenols were administered about 14 hr. after ingestion of the previous day's food ration, when base-line excretion of the metabolites studied was constant. In the early stages of an experiment water was given at a rate of about 100 ml./hr. After 4 hr. the amount was reduced to that necessary to ensure the passing of a sufficient number of urine samples.

RESULTS

For most of the phenols used, studies of the detailed metabolic fate had already been made. Where this information was not available it was confirmed that virtually the whole dose could be accounted for as free phenol, glucuronide and ethereal sulphate. The treatment of analytical data and the calculation of velocity constants were as previously described (Bray *et al.* 1951).

It was found that the rate of excretion of glucuronide was proportional to the body level of the phenol. The velocity constants (k_g) were calculated chiefly by means of the 'tangent' method. The rate of excretion of glucuronide, obtained from the

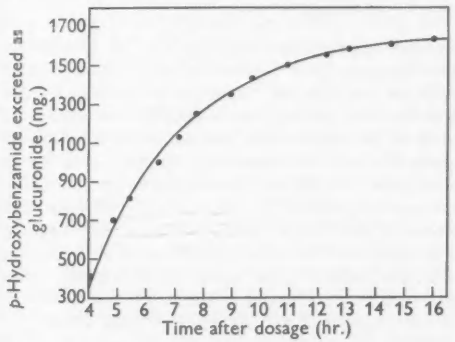

Fig. 1. Excretion of glucuronide by the rabbit after administration of *p*-hydroxybenzamide (1·3 g./kg.). The curve is the theoretical curve for $k_g = 0{\cdot}18$ hr.$^{-1}$ and the points those obtained by experiment.

glucuronide 'difference curve' (see Bray *et al.* 1951, p. 89) was plotted against the body level of the phenol, taken from the total difference curve for all estimated metabolites. The slope of the line gave an approximate value of k_g. A more accurate value was deduced by constructing theoretical glucuronide difference curves for values of k_g close to the approximate one and checking the fit of the experimentally determined points. An example, from an experiment with *p*-hydroxybenzamide, is shown in Fig. 1. In experiments where sulphite or cystine was also administered the sulphate conjugation also follows first-order kinetics (see Bray, Humphris, Thorpe, White & Wood, 1952*c*), so that both the 'log' and 'tangent' methods (see Bray *et al.* 1951) may be used independently for deriving k_g. This can be seen from Table 1 which shows some values for various phenols obtained by the two methods. The mean values of k_g for all the phenols studied are given in Table 2. The application of graphical methods depends upon certain conditions. The rate of excretion should not be taken as the rate of formation if there is appreciable accumulation in the body of the excretion products. It was found for various phenols that the blood level of the glucuronide, even when this metabolite was being excreted rapidly, showed only random deviation (less than ± 4 mg./100 ml.) about the base-line value (average 30 mg./100 ml.). As in the benzoic acid study (Bray *et al.* 1951), only experimental points obtained after absorption was complete, i.e. about 2 hr. after dosage, were used for calculation of velocity constants from the excretion curves.

Table 1. *Comparison of the velocity constants, k_g, for glucuronide conjugation of various phenols in the rabbit derived by 'log' and 'tangent' methods, expressed as hr.$^{-1}$*

(Dose level 0·25 g./kg.)

Compound	k_g found by 'log' method	k_g found by 'tangent' method
Phenol	0·59	0·56
Resorcinol	0·49	0·43
Quinol	0·49	0·47
p-Methoxyphenol	0·52	0·47
N-(*p*-Hydroxyphenyl)-urea	0·20	0·14

The percentage of the dose accounted for as glucuronide and sulphate was usually greater than 80. An exception was *p*-hydroxybenzamide, approximately 25 and 10 % of the dose of which was excreted as the free amide and acid respectively. On the assumption that failure to account for 100 % of the dose was due to the phenol being excreted as undetermined metabolites, the values of k_g derived graphically were corrected to give the value appropriate to 100 % recovery. Thus if the recovery were 85 % and the k_g found were 0·5 hr.$^{-1}$, then the true k_g was taken as 0·42 hr.$^{-1}$. Since the experiments were not continued until all traces of the phenol had been excreted, the 'percentage recovery' referred to is the value of the calculated asymptote, i.e. $E_{max.}$ (Bray *et al.* 1951, p. 90).

DISCUSSION

From the results recorded above it can be seen that conjugation of phenols with glucuronic acid follows first-order kinetics. Table 2 shows that the values of k_g for substituted phenols differ with the substituent, and it includes also P, the probabilities that the

Table 2. *Mean velocity constants, k_g, for glucuronide conjugation of various phenols in the rabbit*

Compound	Weight and influence of substituent radical*	No. of experiments	log $100k_g$ Mean	log $100k_g$ S.D.	P†	k_g‡ (hr.$^{-1}$)
Phenol	1	6	1·748	0·1067		0·56
Group 1						
2:6-Xylen-1-ol	$2 \times 15a$	4	1·543	0·1626	0·01–0·1	0·35
3:4-Xylen-1-ol	$2 \times 15a$	4	1·697	0·1696	>0·1	0·50
Catechol	$17a$	1	1·740	—	>0·1	0·55
Resorcinol	$17a$	2	1·653	0·0273	>0·1	0·45
Quinol	$17a$	2	1·706	0·0482	>0·1	0·51
p-Cyanophenol	$26d$	4	1·721	0·2056	>0·1	0·53
m-Methoxyphenol	$31d$	2	1·902	0·0383	>0·1	0·80
p-Methoxyphenol	$31a$	5	1·733	0·1414	>0·1	0·54
p-Chlorophenol	$35{\cdot}5d$	4	1·615	0·2067	>0·1	0·41
Group 2						
p-Hydroxybenzamide	$44d$	4	1·359	0·1741	<0·1	0·23
N-(*p*-Hydroxyphenyl)-urea	$59a$	4	1·023	0·1414	<0·1	0·11
Hydroxybenzoxazolone	59	4	1·244	0·2122	<0·1	0·18
p-Hydroxybenzenesulphonamide	$80d$	4	1·191	0·3118	<0·1	0·16
p-Benzylphenol	$91a$	4	1·179	0·2037	<0·1	0·15

* Activating radicals indicated by 'a' and deactivating ones by 'd'. (See Hammett, 1937.)

† The probability that the value of log $100k_g$ for the substituted phenol is the same as that for phenol, calculated by Student's 't' test.

‡ Derived from arithmetic mean of log 100 k_g values, i.e. geometric mean of k_g values.

differences from the value for phenol are significant. With one exception all the substituted phenols in group 1 have values of k_g essentially the same as that of phenol itself. 2:6-Xylen-1-ol is the only phenol of this group to have a significantly lower value of k_g. It may be relevant that this phenol has two *ortho* substituents. The phenols of group 2 all give values of k_g significantly smaller than that of phenol. The radical weights of the substituents in group 1 are smaller than those in group 2. Whether the difference in k_g values in groups 1 and 2 is due primarily to the size of the substituent group cannot be determined easily. Considering the *para*-substituted phenols in particular, the inclusion in each group of both strongly activating radicals (e.g. —OCH_3 and —$NHCONH_2$) and strongly de-activating radicals (e.g. —CN and —SO_2NH_2) suggests that the electronic influence is of little importance.

For simplicity, the phenols used for this study were selected because the substituents are metabolically inert, that is, they are modified slightly or not at all *in vivo*. As will be shown in the next paper (Bray *et al.* 1952*c*) sulphate conjugation in the fasting animal takes place at a uniform rate. For phenols containing metabolically inert substituents, therefore, the proportions of the dose excreted as ethereal sulphate and as glucuronide will be determined solely by the values of k_g. The percentages of the dose of a phenol excreted as the two conjugates are complementary, so that if the effect of a metabolically inert substituent is to decrease the percentage glucuronide conjugation, this will be accompanied by a corresponding increase in the sulphate conjugation. Since the percentage of the dose excreted as sulphate is usually less than that excreted as glucuronide, the variation in sulphate excretion will be relatively greater and more noticeable. The effect of substituents upon the percentage conjugation of phenols with sulphate was studied by Williams (1938), but from the foregoing it is clear that for metabolically inert substituents this was indirectly a study of the effect upon glucuronide conjugation. This is discussed further in a later paper (Bray, Thorpe & White, 1952).

SUMMARY

1. The kinetics of the conjugation of phenol, catechol, resorcinol, quinol, *m*- and *p*-methoxy-, *p*-chloro- and *p*-cyano-phenols, and 2:6- and 3:4-xylen-1-ols with glucuronic acid in the rabbit have been studied.

2. The rate of glucuronide formation is proportional to the body level of the phenol.

3. Of the phenols examined, those with small substituent radicals have, with the exception of 2:6-xylen-1-ol, velocity constants, k_g, for glucuronide formation which do not differ significantly from that of phenol itself. Those with substituent radical weight greater than that of *p*-chlorophenol have significantly lower values for k_g.

4. For the *para*-substituted phenols examined the value of k_g does not appear to be related to the electronic influence of the substituent group.

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Kinetic Studies of the Metabolism of Foreign Organic Compounds

4. THE CONJUGATION OF PHENOLS WITH SULPHURIC ACID

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Previous investigations of the conjugation of phenols with sulphuric acid have been chiefly concerned with the percentage of a dose excreted as ethereal sulphate. The observation by various workers (e.g. Tauber, 1895; Hele, 1924, 1931) that simultaneous administration of compounds giving rise to inorganic sulphate *in vivo* increases the percentage sulphate conjugation, together with more direct evidence obtained by the use of radioactive sulphate (Laidlaw & Young, 1948), has shown that the sulphate ion participates in the synthesis and that the amount of sulphate available for conjugation in the normal animal limits the extent of this process. In the investigation now reported the kinetics of the ethereal sulphate conjugation have been studied. In the fasting rabbit the process takes place at a uniform rate, while in the presence of sufficient sulphate the rate is proportional to the body level of the phenol.

METHODS

The animals, their diet, the phenols used and the general plan of experiments and collection of urine specimens were as described in the previous paper (Bray, Humphris, Thorpe, White & Wood, 1952*b*).

Dosage. The experiments performed were of three types: (1) administration of a single dose of a phenol, alone or with a sulphate precursor; (2) administration of repeated small doses of a phenol, alone or with a sulphate precursor; (3) administration of a single dose of a phenol precursor, alone or with a sulphate precursor.

The doses of phenols ranged from 0·4 to 4·0 g. depending on the toxicity of the compound. In experiments of type 2 the phenol (50 mg.) was given each hour for 6 hr. The dose of sulphate precursor varied with the type of experiment. When L-cystine was used 1·5 g. was administered 2 hr. before the dose of the phenol. Larger doses could not be used owing to toxicity (3 g. may cause death within 48 hr.). In experiments of type 1, sodium sulphite ($Na_2SO_3.7H_2O$) was administered in doses of 2 and 1 g. an hour apart, the second dose simultaneously with the phenol. In type 2 experiments, a preliminary dose of sulphite (1 g.) was administered 1 hr. before the first dose of the phenol, and subsequently a small dose (100 mg.) was given each hour at the same time as the phenol throughout the experiment. Sodium sulphite (1 g.) was usually given with the phenol precursor in experiments of type 3.

Table 1. *Rates of excretion of inorganic sulphate after administration of sulphate precursors to rabbits 14 hr. after normal food ration*

Precursor	Dose (g.)	Mean rate (mg. SO_3/hr.) 0–3 hr. after dose	Mean rate (mg. SO_3/hr.) 3–9 hr. after dose
L-Cystine	1·5	50–150	50, 55
L-Cystine	1·0		32, 33
$Na_2SO_3.7H_2O$	2+1		39, 27
$Na_2SO_3.7H_2O$	2		37, 48
DL-Methionine	1	0–25	34, 38
$Na_2S_2O_3.5H_2O$	1		30
Sulphur	1		15, 15
$Na_2SO_4.10H_2O$	0·5		15
Taurine	1		7
Cabbage	100		24, 10
Cabbage	20		17
Pellets	25		19, 7
Bran, oats and cabbage (1:1:5)	70		5, 13

Choice of sulphate precursors. The rate of excretion of inorganic sulphate was determined after the administration of a number of sulphur-containing compounds and diets. The results are given in Table 1. The substances were given

approximately 14 hr. after the rabbits had ingested the normal daily ration of pellets. By this time the excretion of inorganic and ethereal sulphate had fallen to a low and constant level (about 5 mg. inorganic and 1 mg. ethereal SO_3/hr.). Rabbits in this state will be referred to as 'fasting' animals. It can be seen that sulphate precursors vary considerably in their rates of conversion to inorganic sulphate. Sodium sulphite and L-cystine were most effective. With most phenols either could be used, but with *p*-hydroxybenzamide and *p*-chlorophenol only cystine could be given since the simultaneous administration of sodium sulphite caused death even with doses which caused no toxic effects when either was given separately.

Estimation of metabolites. The methods used for urine have been described previously (Bray, Humphris, Thorpe, White & Wood, 1952*a*). Inorganic and ethereal sulphates were determined in blood as follows: blood (about 1 ml.) from a puncture in an ear vein was mixed with potassium oxalate solution (5 ml. 0·5% w/v) in a tared beaker. After weighing, trichloroacetic acid (2·5 ml. 10%, w/v) was stirred in and the mixture filtered. Sulphates were estimated in the filtrate as for urine.

RESULTS

Throughout this investigation it has been assumed that the rate of formation of ethereal sulphate does not exceed the rate of its excretion, i.e. that there is

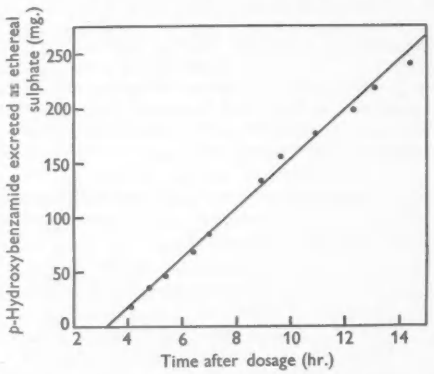

Fig. 1. Excretion of ethereal sulphate after administration of *p*-hydroxybenzamide (3·6 g.) to a fasting rabbit.

no appreciable accumulation of ethereal sulphate in the body. Determination of the blood level of ethereal sulphate after the administration of various phenols has shown that even when rapid excretion was occurring the average increase in the blood level was approximately 2 mg. SO_3/100 ml. This is so close to the experimental error of the method that it is of doubtful significance. After administration of a phenol to a fasting rabbit the rate of ethereal sulphate formation is approximately constant until almost the whole of the dose is eliminated. This is illustrated in Fig. 1 which shows the excretion curve of ethereal sulphate after the administration of *p*-hydroxybenzamide. This constant rate, designated v_s, is of the same order for all phenols examined. The average value for 22 experiments was 12·5 (S.D. ± 5·1) mg. SO_3/hr. (4·5 mg. SO_3/kg./hr.). Since the glucuronide conjugation which is occurring simultaneously proceeds at a rate which is proportional to the body level of the phenol (Bray *et al.* 1952*b*) the ratio of the rate of formation of glucuronide to that of ethereal sulphate (g/s) will decrease throughout the excretion period. This is illustrated in Fig. 2 which was obtained in the same experiment as Fig. 1.

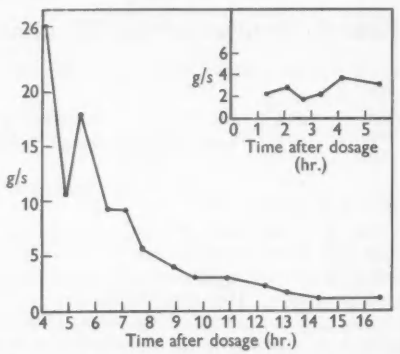

Fig. 2. The relation between the rates of excretion of glucuronide and ethereal sulphate, expressed as g/s, after administration of *p*-hydroxybenzamide to the rabbit (3·6 g.). *Inset:* the same ratio obtained in an experiment in which phenol (0·56 g.) was given together with $Na_2SO_3.7H_2O$ (1 g.) 2 hr. after a dose (2 g.) of the latter.

Table 2. *Excretion of ethereal sulphate in 24 hr. after administration of various phenols with and without sulphate precursor, expressed as percentage of the dose*

(Dose level 0·25 g./kg.)

Compound	Without sulphate precursor	With sulphate precursor
Phenol	15, 16	29, 23, 28, 32
2:6-Xylen-1-ol	12, 9, 16	33, 31
3:4-Xylen-1-ol	13, 11, 12	29, 33
Quinol	18	45
m-Methoxyphenol	12	22
p-Methoxyphenol	10, 15	32, 31, 43, 30
p-Chlorophenol	10, 12	33, 33, 27
p-Hydroxybenzamide	17, 7	40, 36
Hydroxybenzoxazolone	28, 28	40, 52
p-Hydroxybenzenesulphonamide	8, 19	28, 75

When the phenol is administered together with sufficient sulphate precursor different results are obtained; the percentage of the dose excreted as ethereal sulphate is increased (see Table 2) and that as glucuronide is decreased. Ethereal sulphate is formed at a rate proportional to the body level of

the phenol and the ratio g/s* does not steadily decrease throughout the period of elimination. This is illustrated in the inset of Fig. 2 which shows the values for the ratio g/s for phenol administered with sulphite. Fig. 3 shows the excretion curves for ethereal sulphate and glucuronide when *p*-chlorophenol is administered alone and with L-cystine. Fig. 4 shows experimental points obtained with *p*-methoxyphenol plotted together with a theoretical curve calculated for the given value for the first-order velocity constant for ethereal sulphate formation (k_s).*

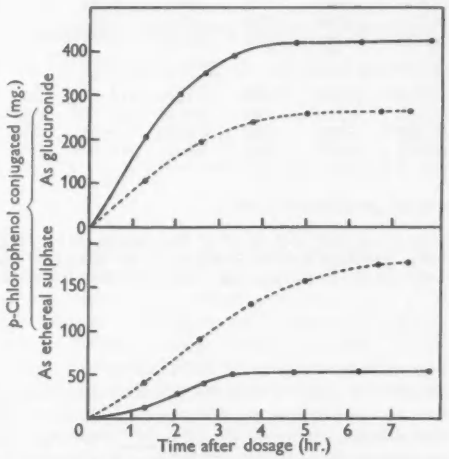

Fig. 3. The effect upon the conjugation of *p*-chlorophenol with glucuronic and sulphuric acids of administration of L-cystine to the rabbit. ●—●, excretion curves after administration of *p*-chlorophenol (0·56 g.) alone; ●---●, excretion curves after administration of *p*-chlorophenol (0·56 g.) 2 hr. after L-cystine (1·5 g.).

An accurate value for k_s could not be derived by means of the graphical methods (Bray, Thorpe & White, 1951) since the maximum amount of sulphate precursor which could be safely administered did not supply sufficient sulphate ion for the conjugation of more than small doses of phenols. This difficulty is accentuated by the more rapid excretion of phenols under conditions where the kinetics of sulphate conjugation follow those of the first-order reactions, so that only a small number of experimental points can be obtained. However, no such limitation exists for the determination of k_g and the constant value of the ratio g/s (designated R), which is characteristic of the first-order kinetics. Both these values can be determined by experiments of types 1, 2 and 3. Clearly R will be numerically equal to the ratio k_g/k_s. It was found that the ratio g/s was steady throughout experiments of type 1 and approximately equal to those obtained in experiments of types 2 and 3 when the ratio of the rate of excretion of inorganic sulphate to that of ethereal sulphate was greater than 2. Consequently, this was adopted as a criterion of adequacy of sulphate ion for conjugation, and values of g/s determined under these conditions were taken as R.

Values of R were obtained from type 1 experiments only when a small dose of a phenol was given with a sulphate precursor and from all experiments

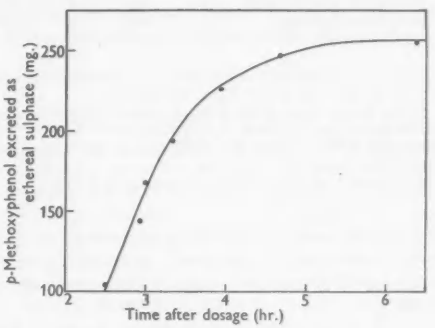

Fig. 4. Excretion of ethereal sulphate by the rabbit after administration of *p*-methoxyphenol and sulphite. The curve is the theoretical one for $k_s = 0{\cdot}60$ hr.$^{-1}$ and the points are those obtained by experiment. Dosage: *p*-methoxyphenol (0·56 g.) with $Na_2SO_3.7H_2O$ (1 g.) 2 hr. after giving $Na_2SO_3.7H_2O$ (2 g.).

of types 2 and 3. These were calculated by taking the mean of the values for the ratio for each urine specimen. In type 1 experiments most of the experimental points relate to high body levels of the phenol; in those of type 2 a low body level was maintained continuously, while in experiments of type 3 the administration of a large dose of a phenol precursor gives a low body level of the phenol which changes only slowly (Bray *et al.* 1952*a*, *c*). The mean value of R for each phenol was calculated by taking the mean of the values for the ratio for each experiment (of all types). In all these calculations the geometric mean was used, i.e. the arithmetic mean of the logarithms of the ratio. Since the value of k_g for each phenol has been given as the geometric mean (Bray *et al.* 1952*b*) the derived values for k_s will also be geometric mean values. Since the variance of both k_g and R for any one phenol may be

* In a preliminary communication (Bray & White, 1951) the first-order velocity constant for ethereal sulphate formation was designated k_e and this ratio G/E. In view of the extension of these studies to other conjugation processes it is preferable to use the general symbol k_s for the first-order velocity constant of the 'second' conjugation reaction (in the present paper this applies to sulphate conjugation) and g/s for the ratio (see Bray, Thorpe & White, 1952*c*).

Table 3. *Mean velocity constants, k_s, for sulphate conjugation of various phenols in the rabbit calculated from R and the k_g values*

Compound	Weight and influence of substituent radical*	No. of exp.	log 100R Mean	log 100R S.D.	log 100k_g† Mean	log 100k_s Mean	log 100k_s S.D.	P‡	k_s§ (hr.$^{-1}$)
Phenol	1	7	2·205	0·1918	1·748	1·543	0·1594		0·35
Group 1									
2:6-Xylen-1-ol	2 × 15a	2	2·231	0·1082	1·543	1·312	0·1589	>0·1	0·21
3:4-Xylen-1-ol	2 × 15a	2	2·233	0·2485	1·697	1·464	0·1816	>0·1	0·29
Resorcinol	17a	3	2·214	0·1657	1·653	1·439	0·0134	>0·1	0·27
Quinol	17a	4	2·023	0·1664	1·706	1·683	0·1593	>0·1	0·48
p-Cyanophenol	26d	3	2·202	0·0687	1·721	1·519	0·1937	>0·1	0·33
m-Methoxyphenol	31d	2	2·284	0·3264	1·902	1·718	0·3239	>0·1	0·52
p-Methoxyphenol	31a	9	1·998	0·2714	1·733	1·735	0·2314	>0·1	0·54
p-Chlorophenol	35·5d	4	2·206	0·2228	1·615	1·409	0·0831	>0·1	0·26
Group 2									
p-Hydroxybenzamide	44d	3	1·017	0·2271	1·359	2·342	0·1459	0·01–0·1	2·2
N-(*p*-Hydroxyphenyl)-urea	59a	9	1·985	0·1182	1·023	1·038	0·0775	0·01–0·1	0·11
Hydroxybenzoxazolone	59	7	1·978	0·0699	1·244	1·266	0·2030	>0·1	0·18
p-Hydroxybenzene-sulphonamide	80d	3	0·859	0·5172	1·191	2·332	0·3877	0·01–0·1	2·1

* Activating radicals indicated by 'a' and deactivating ones by 'd' (see Hammett, 1937).

† From previous paper (Bray *et al.* 1952*b*).

‡ The mean value of log 100k_s was derived from the mean values of log 100k_g and log 100R. The variance of log 100k_s was taken as the variance of log 100R less the variance of log 100k_g. Assuming a normal distribution, the value of P was calculated by comparing the difference in the mean values of log 100k_s for phenol and the substituted phenol with the sum of the variances.

§ Derived from the arithmetic mean of log 100k_s.

derived, the statistical significance of different values of k_s for different phenols may be assessed. The mean values of k_s found for several phenols are given in Table 3.

DISCUSSION

The administration, to a fasting animal, of a sulphate precursor increases the percentage sulphate conjugation of a phenol and transforms the conjugation process from one following zero-order kinetics to one following first-order kinetics. This indicates that the rate of sulphate conjugation is limited by the availability of sulphate and supports the view of the earlier workers already referred to that the sulphate ion is concerned in the conjugation process. Although it has been shown that the ethereal sulphate conjugation follows first-order kinetics with respect to the phenol when a certain amount of inorganic sulphate is available, the effect of a greater excess of sulphate ion could not be determined owing to the limitations of the analytical method.

It can be seen from Table 3 that the values of k_s for substituted phenols of group 1, like those for k_g (Bray *et al.* 1952*b*) do not differ significantly from that of phenol itself. Considering the phenols with larger substituent radicals (group 2), the value of k_s for *N*-(*p*-hydroxyphenyl)-urea is significantly lower than that of phenol, that for hydroxybenzoxazolone is essentially the same, whilst those for *p*-hydroxybenzamide and *p*-hydroxybenzenesulphonamide are very much greater. The values of k_g for these four compounds are all significantly lower than that for phenol. It seems, therefore, that the value of k_s, unlike that of k_g, is not related to the size of the substituent. It can also be seen from Table 3 that the electronic nature of the substituent radicals does not appear to influence the value of k_s.

SUMMARY

1. The kinetics of the conjugation of phenols with sulphuric acid have been studied in the rabbit.

2. In the fasting rabbit the rate of ethereal sulphate formation is independent of the amount of phenol present above a certain body level, but the simultaneous administration of a compound, e.g. sodium sulphite or L-cystine, which is converted to sulphate *in vivo*, changes the kinetics of the process to those of a first-order reaction.

3. The effect of various substituents on the value of the velocity constant for sulphate conjugation was determined and does not appear to be related to the size or electronic influence of the substituent group.

We are indebted to Dr Colin White for advice upon the statistical treatment of our results.

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Kinetic Studies of the Metabolism of Foreign Organic Compounds

5. A MATHEMATICAL MODEL EXPRESSING THE METABOLIC FATE OF PHENOLS, BENZOIC ACIDS AND THEIR PRECURSORS

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Previous papers from this laboratory have recorded the results of studies of the kinetics of the reactions which benzoic acid, phenols and compounds which give rise to them, undergo in the rabbit (Bray, Thorpe & White, 1951; Bray, Humphris, Thorpe, White & Wood, 1952*a*, *b*, *c*). These results have provided a basis for the construction of a mathematical model expressing the quantitative fate of such compounds. This model is in accord with experimental results obtained previously both in this and other laboratories. Experiments with aniline, acetanilide, *o*- and *p*-aminophenols, *o*- and *p*-hydroxyacetanilides and *o*-, *m*- and *p*-hydroxybenzaldehydes have been carried out as further tests of the validity of the model.

THEORETICAL

In this section mathematical expressions are derived representing the fate of compounds such as phenols, benzoic acids and their precursors undergoing metabolic reactions according to a defined pattern of kinetics.

In the previous paper (Bray *et al.* 1952*c*) it was shown that for small doses of a phenol the rate of conjugation with sulphate is proportional to the body level of the phenol, while for large doses the rate is constant and equal to the maximum rate of supply of sulphate ion. In the present paper it will be assumed that the transition from first-order to zero-order kinetics is abrupt. Thus, with increasing body level of the phenol, the rate of conjugation with sulphate increases in proportion until the maximum rate at which sulphate can be supplied is reached. The magnitude of this critical body level will be fixed by the values of the first- and zero-order velocity constants for conjugation. Although such transitions are not encountered in purely chemical systems, our experimental results suggest that they may occur in the rabbit. The mechanism seems to be related to the tendency to maintain a certain blood level of sulphate ion, essentially by means of the kidney. Similar kinetic considerations appear to apply also to glycine conjugation.

The basic mathematical treatment of such a system has already been presented for benzoic acid (Bray *et al.* 1951). In this the critical body level of benzoic acid was equated to zero since the very high value of the first-order velocity constant for glycine conjugation rendered the critical value negligibly small. This treatment is now extended to represent the metabolism of compounds for which this approximation cannot be made. The substance formed, actually or theoretically from the precursor, whether it be a phenol, a benzoic acid or some other compound, is described as the intermediate.

Symbols

A = dose level of precursor; $A_{crit.}$ = critical dose level of precursor; B = dose level of intermediate; $B_{crit.}$ = critical dose level of intermediate; b = amount of intermediate, free and conjugated, at time t; g = amount of glucuronide at time t; s = amount of second conjugate at time t (e.g. an ethereal sulphate for phenols, a hippuric acid for benzoic acids); $m = g + s$; T = time at which critical body level of free intermediate is reached; k_b = first-order velocity constant for conversion of precursor

to intermediate; k_g = first-order velocity constant for glucuronide conjugation; k_s = first-order velocity constant for second conjugation; $k_m = k_g + k_s$; v_s = zero-order velocity constant for second conjugation; G = total amount of glucuronide formed; S = total amount of sulphate formed.

Most of these symbols were used in the study of the kinetics of the conjugation of benzoic acid (Bray *et al.* 1951). The more general symbol v_s now denotes zero-order velocity constant for the second conjugation in preference to the k_h formerly used in reference to hippuric acid formation. Equations 3, 7 and 9 from that paper (p. 95) are quoted using the revised symbols:

$$b = B + A(1 - e^{-k_b t}), \tag{1}$$

$$m = \left(A + B + \frac{v_s}{k_g}\right)(1 - e^{-k_g t}) + \frac{k_g A}{k_g - k_b}(e^{-k_g t} - e^{-k_b t}), \tag{2}$$

$$g_T = A(1 - e^{-k_b T}) + B - v_s T. \tag{3}$$

These equations will now be generalized. When the dose level of the precursor administered is less than a certain value ($A_{\text{crit.}}$) the body level of the free intermediate compound will be less than $(b-m)_{\text{crit.}}$ and the rate of production of the two conjugates will be in the ratio of the respective first-order velocity constants for the conjugation processes. The amount of glucuronide produced will be

$$g = k_g m / k_m,$$

and when $t = \infty$ $\quad G = k_g A / k_m,$

providing these are the only reactions taking place. At the critical body level of the unconjugated intermediate, i.e. at time T, the rate of formation of the second conjugate is given by the equation

$$ds/dt = v_s = k_s(b-m)_{\text{crit.}} = k_s(b_T - m_T),$$

since the body level of unconjugated intermediate at any time is given by the expression $(b-m)$.

Now the general expression for the total amount of glucuronide formed may be derived using the condition that at time T

$$b_T - m_T = v_s / k_s.$$

The glucuronide formed between $t=0$ and $t=T$ is given by

$$[A(1 - e^{-k_b T}) + B - v_s T] - \frac{v_s}{k_s}$$

and that formed after $t=T$ is given by

$$\frac{k_g}{k_m}\left(A e^{-k_b T} + \frac{v_s}{k_s}\right).$$

Thus the total glucuronide formed is given by

$$G = [A(1 - e^{-k_b T}) + B - v_s T] - \frac{v_s}{k_s} + \frac{k_g}{k_m}\left(A e^{-k_b T} + \frac{v_s}{k_s}\right)$$

which, on rearranging and substituting, gives

$$G = A + B - v_s T - \frac{k_s}{k_m}\left(A e^{-k_b T} + \frac{v_s}{k_s}\right), \tag{4}$$

and the total second conjugate formed is given by

$$S = v_s T + \frac{k_s}{k_m}\left(A e^{-k_b T} + \frac{v_s}{k_s}\right). \tag{5}$$

Equation 2 can be extended to represent the metabolism of compounds which are excreted to some extent unconjugated, or which contain substituent groups which undergo modification, if these secondary processes follow first-order reaction kinetics. Where the precursor is excreted unchanged or contains a substituent group which is modified or undergoes any reaction other than the primary reactions considered here, k_b and k_g must be replaced by other first-order velocity constants which comprise the first-order velocity constants of all the individual reactions.

It can be calculated that the critical dose level of a precursor is given by

$$A_{\text{crit.}} = \frac{v_s}{k_s}\left[\frac{k_m}{k_b}\right]^{\left(\frac{k_m}{k_m - k_b}\right)} \tag{6}$$

and of its intermediate by

$$B_{\text{crit.}} = v_s / k_s, \tag{7}$$

assuming instantaneous absorption in both cases.

When equation 2 is simplified to represent the metabolic fate only of an administered precursor, it is not completely accurate, since it is assumed that, for dose levels above $A_{\text{crit.}}$, the body level of the intermediate rises immediately above the critical body level, whereas in fact this increase from zero body level is not instantaneous. While it would be possible to derive the appropriate, more complicated, mathematical expression, in practice this is not necessary, since, for the compounds considered, the increase occupies only a relatively short time and the correction is in any case smaller than that which must be applied to allow for a finite absorption time. If absorption occupies a finite time, the maximum body level of the intermediate which can be attained will be lower, so that the critical dose levels will be somewhat higher than those defined above, and, in particular, the critical dose level, $B_{\text{crit.}}$, of the intermediate will no longer be identical with the critical body level, $(b-m)_{\text{crit.}}$. The correction is, however, relatively small and, for simplicity, the above equations will be applied throughout this paper.

EXPERIMENTAL

The experiments were of the type described previously (Bray *et al.* 1951). The compound under investigation was administered to a fasting rabbit and the urine samples passed during the period of elimination of the dose collected and analysed individually by the appropriate methods previously described (Bray *et al.* 1952*a*). The compounds studied were aniline, acetanilide, *o*- and *p*-aminophenols and *o*- and *p*-hydroxyacetanilides. Results from these experiments are included in Table 1. Similar experiments

Table 1. *The percentage of the dose of various phenols and phenol precursors excreted as glucuronide and ethereal sulphate by the rabbit (unless stated otherwise)*

Compound	Dose level (g./kg.)	% excreted as: Glucuronide *G*	Ethereal sulphate *S*	*G*/*S*	Remarks
Group 1					
Benzene	0·5[1]	11	10	1·1	39% excreted unchanged in expired air[2]
Phenol	0·25[1]	70	19	3·7	—
	0·20[3]	69	15	4·6	$k_g = 0{\cdot}56$ hr.$^{-1}$
Catechol	0·1[4]	70	18	3·9	—
	0·2[3]	76	18	4·2	—
Quinol	0·15[4]	43	30	1·4	—
	0·2[3]	78	18	4·3	Administered to fasting animal
Chlorobenzene	0·15[5]	25	27	0·9	—
o-Chlorophenol	0·17[5]	82	19	4·4	—
p-Chlorophenol	0·17[5]	88	13	6·9	—
	0·23[3]	81	11	7·4	—
4-Chlorocatechol	0·15[6]	87	15	5·7	—
Anisole	0·3[3]	48	29	1·7	$k_b = 0{\cdot}11$ hr.$^{-1}$
p-Methoxyphenol	0·38[3]	69	13	5·3	$k_g = 0{\cdot}54$ hr.$^{-1}$
Aniline	0·07[7]	0	100	0	Rats
	0·2[8]	70	28	2·5	—
	0·2[9]	59	20	2·8	—
	0·2[9]	68	36	1·9	Sulphite given with dose. $k_b = 0{\cdot}27$ hr.$^{-1}$
o-Aminophenol	0·21[8]	42	25	1·7	—
	0·25[9]	60	22	2·7	—
p-Aminophenol	0·9[7]	100	0	∞	Rats
	0·25[8]	43	18	2·5	—
	0·25[9]	55	18	5·5	Administered to fasting animal
Group 2					
Phenylurea	0·2[9]	39	25	1·6	—
N-(*p*-Hydroxyphenyl)-urea	0·2[9]	34	25	1·4	$k_g = 0{\cdot}11$ hr.$^{-1}$
Benzoxazolone	0·2[9]	53	26	1·7	$k_b = 0{\cdot}29$ hr.$^{-1}$
Hydroxybenzoxazolone	0·2[9]	43	28	1·9	$k_g = 0{\cdot}18$ hr.$^{-1}$
Acetanilide	0·25[8]	70	12	5·8	—
	0·25[9]	58	10	5·8	$k_b = 0{\cdot}43$ hr.$^{-1}$
	0·25[9]	80	29	2·7	Sulphite given with dose
o-Hydroxyacetanilide	0·28[8]	39	20	2·0	—
	0·25[9]	50	22	2·3	—
p-Hydroxyacetanilide	0·28[8]	63	10	6·3	—
	0·25[9]	81	6	13·5	Administered to fasting animal
	0·25[9]	77	6	12·8	k_g 0·50 hr.$^{-1}$

(1) Porteous & Williams, 1949.
(2) Parke & Williams, 1950.
(3) Bray *et al.* 1952*a*, *b*, *c*.
(4) Garton & Williams, 1949.
(5) Spencer & Williams, 1950.
(6) Dodgson & Williams, 1949.
(7) Elson *et al.* 1946.
(8) Smith & Williams, 1949.
(9) This paper.

p-Hydroxybenzenesulphonamide has been omitted from group 2 since its theoretical precursor, benzenesulphonamide, is hydroxylated to such a small extent in the rabbit that values for glucuronide and ethereal sulphate would be unreliable.

Table 2. *Excretion products of* o-, m- *and* p-*hydroxybenzaldehydes in the rabbit*

(Dose level 0·4 g./kg. Ranges given in parentheses and number of experiments as superior figures.)

	% of dose excreted as: Ether-soluble acid	Ester glucuronide	Ether glucuronide	Ethereal sulphate
o-Hydroxybenzaldehyde	75 (64–81)[3]	18 (9–29)[6]	9 (0–19)[6]	3 (2–4)[3]
m-Hydroxybenzaldehyde	75 (66–90)[6]	9 (8–9)[6]	9 (2–13)[6]	7 (6–9)[3]
p-Hydroxybenzaldehyde	67 (51–86)[8]	4 (0–6)[8]	16 (10–21)[8]	9 (9–10)[3]

were carried out on human subjects using benzoic acid. Some further experiments with 24 hr. samples of urine were also carried out with *o*-, *m*- and *p*-hydroxybenzaldehydes, using the analytical methods described by Bray, Ryman & Thorpe (1947). The results obtained are given in Table 2.

DISCUSSION

The mathematical model gives quantitative values for the proportions of the dose excreted as the appropriate metabolites in terms of reaction velocity constants and the dose level. The model reveals certain characteristic features of the metabolic system which may be compared with experimental data. For much of the data in the literature comparison must be qualitative, but, where velocity constants have been evaluated by investigations of the kinetics, comparisons can be made quantitative. In the following sections experimental data from kinetic studies reported in this and previous papers and from 24 hr. investigations in this and other laboratories are considered in relation to the mathematical model.

Variation in dose level

When an administered compound is excreted only as two conjugates, the proportions of the dose appearing in the two forms are complementary.

Table 3. *Variation in ethereal sulphate excretion by rabbits after administration of phenol and p-hydroxybenzenesulphonamide at different dose levels*

Compound	Dose level (mg./kg.)	Percentage of dose excreted as ethereal sulphate	
		Found*	Calculated†
Phenol	25	41	37
	50	37	28
	100	18	20
	200	18	13
	500	19	11
p-Hydroxybenzene-sulphonamide	100	53	56
	250	44	43
	500	33	33
	750	27	23

* Values for phenol given by Williams (1938) and for *p*-hydroxybenzenesulphonamide by Sammons *et al.* (1941).
† Uncorrected for absorption time.

Equations 4 and 5 show that these proportions are a function of the dose level (A and/or B) for dose levels above the critical values $A_{crit.}$ and $B_{crit.}$ (equations 6 and 7). The proportion excreted as that conjugate formed only by a first-order reaction (glucuronide) increases with increasing dose level, while that formed by a zero-order reaction (ethereal sulphate, hippuric acid) decreases. It is most convenient to consider the conjugate excreted in smaller amount, since this shows a greater relative variation.

We have already shown that the proportion of a dose of benzoic acid excreted as benzoylglucuronide is greater for higher dose levels (Bray *et al.* 1951). Variation in the extent of ethereal sulphate formation at different dose levels was recorded for phenol by Williams (1938) and for *p*-hydroxybenzenesulphonamide by Sammons, Shelswell & Williams (1941). These data are compared in Table 3 with the values calculated by means of equation 5, using values of constants (v_s, k_g, k_s) derived experimentally for the rabbit in previous kinetic studies (Bray *et al.* 1952*b*, *c*), i.e. $v_s = 4{\cdot}5$ mg. SO_3/kg./hr.; for phenol $k_g = 0{\cdot}56$ hr.$^{-1}$, $k_s = 0{\cdot}35$ hr.$^{-1}$; for *p*-hydroxybenzenesulphonamide $k_g = 0{\cdot}16$ hr.$^{-1}$, $k_s = 2{\cdot}1$ hr.$^{-1}$. It is clear that the observed effects of varying the dose levels are close to those calculated from equation 5.

Effect of administration as precursor

An intermediate, e.g. a phenol or a benzoic acid, gives rise to excretion products which are the same whether it is administered as such or formed *in vivo* from a precursor, but the proportions of the metabolites usually differ considerably for equimolecular doses. For dose levels below the critical ones the proportions of the intermediate excreted as different conjugates do not vary. The critical dose for the intermediate ($B_{crit.}$) is defined in equation 7, and for the precursor ($A_{crit.}$) in equation 6. For dose levels above $A_{crit.}$ and $B_{crit.}$ the considerations discussed in the previous section apply. It so happens that for most precursors and their intermediates, the quantitative metabolic fate of which has been determined, the order of dose level low enough to be without obvious toxic effect and yet high enough to permit accurate estimation of excretion products greatly exceeds $B_{crit.}$ and is comparable with $A_{crit.}$. When this condition holds, the ratio of the amount of glucuronide to the amount of the other conjugate will be smaller if the precursor is administered than if the intermediate is administered.

Although we have not yet investigated the kinetics of the metabolic reactions of substituted benzoic acids and their precursors in detail, there are indications from other studies that the same mathematical model is applicable to certain of these compounds. Administration of the chloro- and fluorobenzoic acids (Bray, Clowes, Thorpe, White & Wood, 1952) gives rise to the excretion of a considerable proportion of the dose as unconjugated acid, but it was shown that the ratio of the proportion of the dose excreted as the glycine conjugate to that excreted as glucuronide was greater after administration of the amide than after the acid. That the ratio is greater for amide than for acid is a characteristic of the model, whether the rate of excretion of

unconjugated acid increases or decreases with decreasing body level, while the relation between the percentages of a metabolite excreted is only characteristic if the excretion of unconjugated acid follows first-order kinetics. We have not investigated in any detail the relation between rate of excretion of an unconjugated compound and its body level, but have noted that the rate usually decreases with decreasing body level, and may, therefore, be considered to follow, approximately, first-order reaction kinetics. When little excretion of the unconjugated intermediate occurs, variations in the proportion of the dose excreted as the minor conjugate forms the most sensitive index for comparison. This is seen in the results of a study of *m*- and *p*-methyl-substituted toluenes, benzamides and benzoic acids (Bray, Humphris & Thorpe, 1949; Bray, Thorpe & Wood, 1949). The percentage of doses of these compounds excreted as *m*- and *p*-toluylglucuronides were, for the *meta* isomers, 2, 9 and 26 respectively, and, for the *para* isomers, 1, 4 and 14. These figures are compatible with equation 4 and show that the methyl group is converted more slowly than the carbamyl group, as was found for toluene and benzamide (Bray *et al.* 1951).

Data from the literature relating to the excretion of ethereal sulphate and glucuronide after administration of various phenols and phenol precursors are summarized in Table 1, in which the compounds have been placed in two groups on the same basis as previously (Bray *et al.* 1952*b*, *c*), i.e. compounds in group 1 have small substituent radicals.

It can be seen that for compounds of group 1 (benzene, chloro-, amino- and methoxy-benzenes and their metabolic hydroxylation products) the value of the ratio G/S (the ratio of the percentages of the dose excreted as glucuronide to that excreted as sulphate) for the precursors is smaller than for the phenols. Apparent exceptions are the findings of Garton & Williams (1949) for quinol (a hydroxylation product of benzene) and of Smith & Williams (1949) for *p*-aminophenol (a hydroxylation product of aniline). Their values for G/S are of the same order as those for benzene and aniline, respectively, whereas we found that these phenols, like the other phenols of group 1, gave values for G/S of approximately 5. As will be seen later (Table 4), this difference cannot be attributed to the slightly different dose levels used, but may be due in part to the fact that the animals used in our experiments were fasting. The results of Garton & Williams might be expected if their rabbits ingested their daily food ration at a time close to that of dosage. The effect of diet and time of feeding is discussed in a later section.

Those phenols of group 1 which have been investigated in detail have values for k_g and k_s which are close to those of phenol itself (Bray *et al.* 1952*b*, *c*). The values of k_b for the precursors in group 1 are low. That for anisole is 0·11 hr.$^{-1}$; benzene (Porteous & Williams, 1949) and chlorobenzene (Spencer & Williams, 1950) are excreted at least as slowly as anisole. For compounds of group 1 the values of G/S for phenols and precursors at the dose levels employed must be widely different, and small variations in the dose levels will not obscure this difference. This can be seen in Table 4 which shows the values of G/S calculated using equations 4 and 5 at different dose levels of anisole and *p*-methoxyphenol, compounds for which the values of the appropriate constants are known.

Table 4. *Calculated values for G/S after administration of anisole and* p*-methoxyphenol to rabbits at varying dose levels*

Dose level (g./kg.)	Anisole	*p*-Methoxyphenol
0·1	1·0	3·5
0·2	1·2	5·2
0·3	1·7	7·1
0·4	2·2	8·5
0·5	2·7	10·0

The values for aniline and *p*-aminophenol in the rat given by Elson, Goulden & Warren (1946) require comment. These authors stated that 'aniline is excreted exclusively as ethereal sulphate, whereas the equivalent dose of *p*-aminophenol is excreted entirely as glucuronate'. In view of this they suggested that ethereal sulphate could be formed in the rat 'directly in the aromatic nucleus without preliminary formation of free phenol compounds'. It seems unlikely that there is a qualitative difference between the metabolism of phenols in the rabbit and rat, although quantitative differences might be expected. We have found, for example, that in the rabbit the value of G/S for a small dose of *p*-hydroxybenzenesulphonamide (0·2 g./kg.) is 0·11, while for a large dose (2 g./kg.) it is 7·9. When repeated very small doses (0·018 g./kg./hr.) are administered together with sodium sulphite, a method of dosage approximately corresponding to the slow formation of the phenol from a precursor *in vivo*, the amount of glucuronide is not detectable. In the rabbit the value of k_s for *p*-hydroxybenzenesulphonamide is considerably greater than that of k_g. It is reasonable to suggest that the results of Elson *et al.* are of this type and that they failed to detect the minor metabolites due to limitations of their experimental methods. If this explanation of their results is correct their inference of direct sulphation of aniline is no longer valid.

For the phenols included in group 2 of Table 1, k_g is small and the value of G/S increases only slightly as the dose level increases, so that there is little difference between the values of the ratio for phenol

and precursor. For instance, the calculated values of G/S for phenylurea and *p*-hydroxyphenylurea at the dose levels cited are 1·3 and 1·6 respectively. The experimental results are therefore as would be expected.

Acetanilide appears to differ from all the precursors considered so far, in that the value of G/S is unusually high for the dose level cited. This is not because the value of k_s for *p*-hydroxyacetanilide is abnormally low, since the simultaneous administration of a sulphate precursor with acetanilide reduces the value of G/S to a more usual value (2·7), while the value of k_g (0·5 hr.$^{-1}$) is of the same order as for

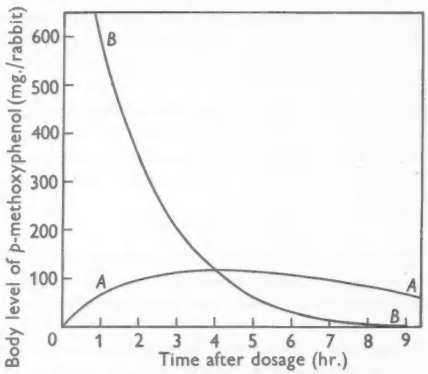

Fig. 1. Calculated body level of *p*-methoxyphenol resulting from the administration of anisole (0·3 g./kg.) and of *p*-methoxyphenol (0·34 g./kg.) to the rabbit, assuming instantaneous absorption. *A–A*, anisole; *B–B*, *p*-methoxyphenol.

the phenols of group 1. The high value of G/S is due to the high value of k_b (0·43 hr.$^{-1}$). This has the effect of reducing the critical dose level so that $A_{crit.}$ is much lower than the dose level used. Since k_g is high, the value of G/S for both acetanilide and *p*-hydroxyacetanilide will increase greatly with increasing dose level, and the actual value of G/S for both compounds will be relatively high. Large variations in the higher values of G/S can occur through small variations in the value of v_s, so that different large values of the ratio for a compound may be expected. The data for acetanilide and *p*-hydroxyacetanilide thus conform to the model.

From the results quoted in this and the preceding paper (Bray *et al.* 1952*c*) it is clear that the administration of a precursor is equivalent to the continuous administration of its hydroxylation product at a low dose level. This is illustrated in Fig. 1 which shows the calculated variations in the body level of *p*-methoxyphenol resulting from the administration of 0·3 g./kg. anisole and of 0·34 g./kg. *p*-methoxyphenol assuming instantaneous absorption. The values of constants substituted in equations 1 and 2 for this calculation are as follows:

$v_s = 4{\cdot}5$ mg. SO_3/kg./hr.; $k_b = 0{\cdot}11$ hr.$^{-1}$;
$k_g = 0{\cdot}54$ hr.$^{-1}$; $k_s = 0{\cdot}54$ hr.$^{-1}$.

Thus the magnitude of the dose of an intermediate having the same metabolic fate as a given dose of its precursor (in the fasting animal) is not the equimolecular dose. For instance, the dose of *p*-methoxyphenol having the same metabolic fate as 1·15 g. of anisole is not 1·0 g. but 0·17 g. of *p*-methoxyphenol.

Species differences

Quantitative differences may occur in the metabolic fate of a given compound in different species, even when the metabolites are identical. The velocity constants found in these kinetic studies must comprise both fundamental chemical constants and parameters characteristic of the organism. While the chemical constants of the enzyme system might be assumed to be identical in all species, the parameters relating to factors approximately constant within a species (e.g. amount or concentration of enzyme) may differ. Thus a reaction may follow similar kinetics in different species, the quantitative differences being due to variations in the values of velocity constants (e.g. the metabolism of aniline and *p*-aminophenol in rabbit and rat, p. 427). In a previous paper (Bray *et al.* 1951) the different proportions of glucuronide excreted by the rabbit, dog, pig and man in response to a dose of benzoic acid were related to the rates of availability of glycine (i.e. the zero-order velocity constant for the formation of hippuric acid) in the species. In these calculations it was assumed that the first-order velocity constant for glucuronide formation (k_g) was the same for all four species, and it was shown that the variation in glycine availability was sufficient to account for species difference together with the dose-level effect, since benzoic acid was given to man at a much lower dose level than to the other species. Approximate values for k_g have now been calculated for the dog and the pig using data of Quick (1931) and Csonka (1924). These are 0·09 and 0·06 hr.$^{-1}$ respectively. The value for the rabbit is 0·08 hr.$^{-1}$ (Bray *et al.* 1951). Experiments on two human subjects gave 0·03 hr.$^{-1}$, a value which differs appreciably from that for the rabbit. Substitution of this value in equation 3 gives a value of 4 for the percentage of a dose of benzoic acid excreted as glucuronide (dose level 0·043 g./kg.). This compares more favourably with the value of 5, found experimentally, than with the value of 9% previously calculated (Bray *et al.* 1951, Table 2).

Effect of substituents

If a substituent is introduced into the benzene nucleus of an aromatic compound, the metabolic fate of the substituted compound may differ from that of the parent compound since (1) the velocity constants of metabolic reactions involving groups in the parent compound may be different in the substituted compound; (2) the substituent group may itself be modified; (3) the rate of excretion (usually by the kidney) may be different for the two compounds. The relative contributions of these three factors cannot be assessed when the only data available are those derived from a study of 24 hr. urine specimens (i.e. the percentages of the dose excreted as different metabolites). The theoretical value of such results is limited unless they can be interpreted with the help of kinetic data. The greater experimental difficulties in the kinetic method are outweighed by the greater scope in interpretation of the results obtained. As an example some early observations on ethereal sulphate formation carried out in this laboratory (Williams, 1938) may be given a different interpretation. Williams studied the effect of various substituents upon the extent of the sulphate conjugation of phenol in the rabbit. With phenol itself 20% of the dose was conjugated as sulphate, but with eleven *para*-substituted phenols, values ranging from 7 to 33% of the dose were obtained. (Most of these values have been confirmed subsequently by other workers in this laboratory.) The deviations from 20%, the value for unsubstituted phenol, were attributed by Williams to the influence of the substituent group upon sulphate conjugation. For phenols substituted with inert groups in the *para* position, we have shown that sulphate conjugation normally takes place at a uniform rate, and that the proportion of a dose of a phenol excreted as ethereal sulphate depends primarily upon the value of k_g for dose levels of the order of 0·25 g./kg. (Bray *et al.* 1952*b*). There is no reason to suppose that this applies only to inert substituents since *p*-hydroxybenzamide, the carbamyl group of which is hydrolysed to some extent, behaves similarly. For phenols with substituent groups which are extensively modified in the body the extent of sulphate conjugation will depend not only on the k_g for the administered phenol (and its phenolic metabolites), but also on the rates of reactions by which the substituent groups are modified. Some of the compounds showing the greatest departure in the extent of sulphate conjugation from that of phenol itself may now be considered. Only 7% of a dose of *p*-hydroxybenzoic acid forms ethereal sulphate. This is probably due to the fact that the acid itself is readily excreted by the kidney rather than to influence of the carboxyl group upon sulphate conjugation. The substitution of benzoic acid with chloro and fluoro radicals reduces the total conjugation (Bray *et al.* 1952) probably for the same reason. Williams found that only 12% of a dose of *p*-hydroxybenzaldehyde formed ethereal sulphate. This need not be attributed to the direct influence of the aldehyde group upon the sulphate conjugation process, since *p*-hydroxybenzaldehyde, which would be expected to behave like phenol itself, is largely metabolized to *p*-hydroxybenzoic acid (Table 2). Of the phenols examined by Williams, *p*-hydroxybenzamide formed the greatest proportion of ethereal sulphate (33% of the dose). Such a high percentage is a necessary consequence of the low value for k_g (0·23 hr.$^{-1}$, Bray *et al.* 1952*b*). The value for ethereal sulphate formed from this compound calculated from equation 5 is 30%. These examples show that the variations in sulphate conjugation may be due to the influence of the substituent group on glucuronide synthesis and not on ethereal sulphate synthesis, to metabolic modification of the substituent group or to the readiness of excretion of the substituted phenol. Correct interpretations of metabolic processes are unlikely to be made from data from 24 hr. studies alone.

Effect of diet

When a reaction follows zero-order kinetics and its rate is determined by the availability of the conjugating moiety, then ingestion of a compound giving rise to the latter will increase the rate of the reaction. It is immaterial whether the administered compound is a simple chemical compound or a constituent of the normal diet. Thus the extent of the conjugation in question, and, therefore, also of the complementary conjugations, will depend upon the nature of the diet. Griffith & Lewis (1923) found that the administration of glycine increased the rate of hippuric acid formation in the rabbit. Our own experiments (Bray *et al.* 1951) confirmed this and showed also that the extent of glucuronide conjugation was decreased. Other workers (e.g. Csonka, 1924) have examined the effect of administering various proteins with benzoic acid and found that their efficacy in increasing hippuric acid excretion could be correlated with their glycine content. Since the first-order velocity constant for glycine conjugation appears to be high, this conjugation process may in practice be considered always to follow zero-order kinetics with respect to benzoic acid, and the zero-order velocity constant (v_s) may be equated to the rate of supply of glycine. The metabolic fate of benzoic acid or of its precursors found in different investigations will not necessarily be the same, since the availability of glycine will depend on the type of food given and on the relative times of administration of the dose and ingestion of the normal diet. It should be noted that the time at

which food is supplied is not necessarily related to the time at which it is ingested since anorexia is often one of the first symptoms of toxicity.

The availability of sulphate will also influence the ethereal sulphate conjugation of phenols. If certain sulphur compounds are administered sulphate conjugation can be made to follow first-order reaction kinetics, but the rate of production of sulphate from foodstuffs is so small (see Table 1, Bray *et al.* 1952*c*) that, except at very low dose levels of a phenol, the reaction follows zero-order kinetics, the zero-order velocity constant (v_s) varying with the nature of the food and the time of its ingestion relative to that of the dose.

SUMMARY

1. A mathematical model is derived for metabolic systems in which a precursor is converted into an intermediate by a first-order reaction, the intermediate then being conjugated by two processes, one of which is first order, and the other first order below a certain body level and zero order above that level.

2. This model is shown to apply to the metabolic fate of certain benzoic acids and phenols and certain of their precursors.

3. The effects of variation in dose level, nuclear substitution of aromatic compounds, and the composition of the diet are discussed.

4. Various data obtained from 24 hr. studies are discussed in terms of the mathematical model.

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Liver Alcohol Dehydrogenase and Ester Formation

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As a result of his observation of clinical cases of methanol poisoning, Røe (1943) formed the opinion that the toxic effects of methanol in man were minimized by the simultaneous ingestion of ethanol. He suggested that ethanol might act as an antidote by virtue of 'the capacity of ethyl alcohol to displace methyl alcohol from the inner surfaces of cells'. The characteristic toxic effects of methanol have, however, generally been attributed to the action of its oxidation products rather than to methanol itself, and it seemed possible that the beneficial effect of ethanol might be the result of competitive inhibition of an early enzymic stage in the metabolism of methanol. Experiments carried out on human subjects have shown clearly that the metabolism of methanol can be strongly impeded at its earliest stage by the administration of ethanol, and *in vitro* tests have provided evidence of competitive inhibition by ethanol of the oxidation of methanol to formaldehyde by liver alcohol dehydrogenase (Zatman, 1946; Leaf & Zatman, 1952). The *in vitro* tests referred to were carried out with horse-

liver alcohol dehydrogenase, prepared according to Lutwak-Mann (1938), and the extent of methanol oxidation in the mixed substrate (methanol + ethanol) system was assessed by colorimetric estimation of the accumulated formaldehyde. The observation of Lutwak-Mann that alcohol dehydrogenase preparations of this type always retain appreciable aldehyde mutase activity has been confirmed. It thus became evident that further reaction of formaldehyde under the influence of mutase might obscure the issue when a more detailed examination of substrate competition in the alcohol dehydrogenase system was planned. Although Lutwak-Mann refers to formaldehyde as susceptible to dismutation under the influence of liver aldehyde mutase, no quantitative study of the reaction of this substrate appears to have been recorded. It was therefore decided to examine the behaviour of formaldehyde, alone and in the presence of methanol and ethanol, in these mutase-containing alcohol dehydrogenase preparations. This led to the recognition of a new and unexpected reaction, with which the present paper is chiefly concerned.

A preliminary account of some of the work has already been published (Kendal & Ramanathan, 1951).

METHODS

Manometric estimation of acid production. Dixon & Lutwak-Mann (1937) found that the mutase reaction was most conveniently studied manometrically in bicarbonate buffer, since under their conditions 2 mol. acetaldehyde gave a theoretical yield of 1 mol. ethanol and 1 mol. acetic acid. In the present work, formic acid production was followed manometrically in the Warburg apparatus at pH 7·4. The main vessel contained coenzyme I (CoI), enzyme, the alcohol when desired and 0·3 ml. 0·15 M-$NaHCO_3$; aqueous formaldehyde was placed in the side arm. Total liquid volume, 2·0 ml. Equilibration took place in a bath at 37° and gassing was with N_2 containing 5 % (v/v) CO_2 in the usual way for 15 min. Dixon & Lutwak-Mann, in similar experiments with acetaldehyde, found that about 20 % of the volatile aldehyde was lost during the passage of the gas stream, and made an allowance for this. It was found under our conditions, by chemical estimation of the formaldehyde present at the end of the gassing and equilibration, that the loss of formaldehyde was rather variable, but was not more than about 5 %. The variability of this loss as between different manometers is presumed to be the result of (*a*) differences in rates of gas flow, and (*b*) inequalities in surface area of formaldehyde solution exposed, due to considerable differences in shape of side arms in the series of flasks. Dixon & Lutwak-Mann found it necessary to correct for the CO_2-retention caused by the rather high protein content of their media (up to 30 mg. of enzyme preparation/ml.). At the maximum enzyme concentrations used in this work (2 mg./ml.), tests showed CO_2-retention to be negligible. None of the reaction mixtures gave a measurable O_2 uptake under aerobic conditions.

Estimations of formaldehyde and formate. Residual formaldehyde and accumulated formate were estimated at the end of the manometric period. When formaldehyde only was to be estimated, the reaction was stopped by the addition of 1 ml. 4 % (w/v) trichloroacetic acid (TCA) to 2 ml. of reaction mixture. After waiting 5 min. for completion of flocculation of the precipitated protein, 3 ml. water were added and the suspension centrifuged. An appropriate volume of the clear supernatant was then taken for colorimetric estimation of formaldehyde using the method of Leaf & Zatman (1952). Test estimations with known amounts of formaldehyde, in which the TCA was added before the formaldehyde, or in which CoI was omitted from the system, showed that the recovery was quantitative.

Formate was determined according to Bastrup (1947), and it was found necessary, when formate estimation was desired after manometric observation, to run manometer flasks with identical contents in triplicate to provide sufficient pooled reaction mixture for satisfactory estimation. Preliminary experiments showed that the formate estimation could not be reliably carried out on samples containing TCA. The enzyme reaction was therefore stopped by the addition of 0·2 ml. 6 N-HCl to each Warburg flask, and the contents of three identical flasks were pooled and made up to 20 ml. with water. Of this, 1 ml. was then treated with TCA and used for formaldehyde estimation as already described. The remainder was transferred to the Bastrup distillation apparatus and formate determined. Tests with known amounts of formate showed a 97 % recovery in estimations on samples containing 0·5–8·0 mg. formic acid, and the correction suggested by this finding was applied. The presence of formaldehyde in the sample did not influence the formate determination.

Colorimetric estimation of formic ester. The method developed by Keenan (1945) for the estimation of ethyl acetate in methanolic solution, as modified by Hestrin (1949) and Peel (1951) for use with aqueous solutions, was found to be applicable to the estimation of formic ester. It depends upon the quantitative conversion of the ester into the corresponding hydroxamic acid by alkaline hydroxylamine, followed by measurement of the colour intensity of the ferric complex of the hydroxamic acid in acid solution. The procedure of Peel was adopted, after modification necessitated by the presence of varying amounts of formaldehyde in the samples to be assayed. Peel, who estimated ethyl acetate in the presence of acetaldehyde, found that the latter in high concentration (0·1 M) caused a slow increase in colour intensity for some minutes after the completion of the addition of the reagents. By taking successive colorimeter readings at timed intervals he was able to deduce the zero-time value by extrapolation. With formaldehyde, this effect is very much greater, even in much lower concentration (0·01 M), and a simple extrapolation to zero time is not possible. It was found, however, that the colour-producing effects of methyl formate and formaldehyde were strictly additive and that, if the order of addition of reagents were so altered as to prevent coincidence of ester and hydroxylamine in alkaline solution, the result was a complete suppression of colour formation by the ester without any effect on colour formation due to formaldehyde. These findings made it possible to develop a satisfactory procedure for the estimation of formic ester in reaction mixtures containing unknown amounts of formaldehyde, as follows.

Two identical 2 ml. samples are required for each estimation. Into each of two tubes 4·0 ml. M-NH_2OH.HCl is measured. To one of these tubes are then added in the order given, mixing after each addition, (*a*) 2·0 ml. sample, (*b*)

2·0 ml. 2·5 N-NaOH; after 5–10 min., (*c*) 1·0 ml. 5·6 N-HCl and (*d*) 1·0 ml. 15% (w/v) $FeCl_3$ in 0·2 N-HCl. To the other tube the same additions are made, but in the order (*b*), (*c*), (*a*), (*d*). In the first tube, colour production is due to ester and formaldehyde additively, in the second tube to formaldehyde alone. Enzyme protein gives a turbidity in the coloured solutions which must be removed by centrifuging; 5 min. at 3200 rev./min. was found to suffice. Exactly 15 min. after the addition of the $FeCl_3$ reagent, readings are taken in a photoelectric colorimeter (Evans Electroselenium Ltd.) with Ilford filter 624. The two readings are converted into methyl formate equivalents by the use of a calibration curve, and the difference between them gives the amount of methyl formate in the sample. The calibration curve is prepared by the use of the above procedure with freshly prepared aqueous standard solutions of methyl formate. The curve was linear in the range used, which was up to 15 μmoles ester/2 ml. sample. The presence of phosphate in the samples has a significant effect in diminishing the slope of the curve. The samples analysed in the present work were either phosphate-free or 0·1 M with respect to phosphate, and in the latter case a calibration curve prepared with standards also containing 0·1 M-phosphate was used. Ethyl formate is not distinguished in any way from methyl formate in this method.

Enzyme preparations. The enzyme source was horse liver. The alcohol dehydrogenase was obtained according to the instructions given by Lutwak-Mann (1938) for her 'acetone preparation'. Most of the experiments were carried out with this material, but similar results were also obtained with an aldehyde mutase preparation made according to Dixon & Lutwak-Mann (1937), with the omission of the final $Ca_3(PO_4)_2$ adsorption stage in the procedure of these authors. Neither preparation exhibited any alcohol dehydrogenase or mutase activity in the absence of added Co I. Formaldehyde in the concentrations used (usually 1 mg./ml.) did not appear to have any inactivating effect upon the enzyme. Marked inactivation was, however, noted on an occasion when the enzyme was incubated with the formaldehyde for a short period before the addition of Co I, and it seems probable that the latter has a protective effect.

Coenzyme I preparations. The instructions of Williamson & Green (1940) were followed. Spectrophotometric assay, as described by Slater (1950), indicated a purity of about 35%.

RESULTS

In preliminary work suitable conditions for manometric experiments, as regards enzyme and coenzyme concentration, were determined. The curves of Fig. 1 show the course of acid production from formaldehyde in the presence of enzyme, and the effect upon this of variation in Co I concentration. In a series of experiments of this kind it was found that whilst activity in the absence of added Co I was negligible, the introduction into the medium of 0·125 mg./ml. of the coenzyme preparation exerted nearly a maximal effect. In all subsequent experiments, therefore, the concentration of the coenzyme preparation was kept constant at 0·25 mg./ml.

Effect of methanol and ethanol on acid formation and formaldehyde disappearance. Experiments limited to manometric observation, in which mutase activity was assessed only by acid formation, showed the latter to be markedly inhibited by ethanol and but little affected by methanol, when the alcohols were present in 0·25 M concentration. A possible explanation of this result suggested itself, based on the view that the mutase is simply a

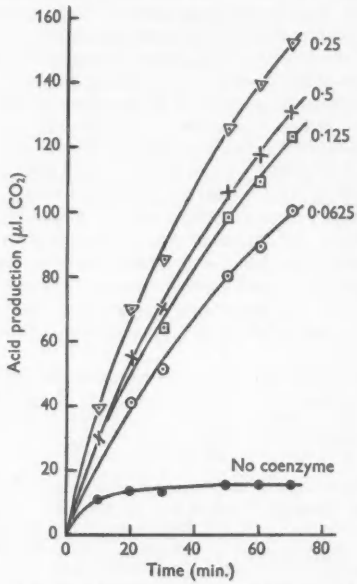


Fig. 1. Relation of mutase activity to Co I concentration. Manometric observation of acid production from formaldehyde. The reaction mixtures contained 1 mg./ml. of enzyme preparation and 0·5 mg./ml. formaldehyde. Co I concentrations (mg. preparation/ml.) as indicated by the curves.

coenzyme-linked system of alcohol and aldehyde dehydrogenases. In this view, the component reactions are

$$H.CHO + H_2O + Co\,I \rightarrow H.COOH + Co\,I\,H_2 \quad (1)$$

$$H.CHO + Co\,I\,H_2 \rightleftharpoons H.CH_2OH + Co\,I \quad (2)$$

catalysed by aldehyde dehydrogenase and alcohol dehydrogenase respectively. The simultaneous presence of ethanol might permit the alcohol dehydrogenase-catalysed reaction

$$C_2H_5OH + Co\,I \rightleftharpoons CH_3CHO + Co\,I\,H_2 \quad (3)$$

to compete with reaction (1) for the available Co I and, to a degree dependent upon the success of this competition, to diminish the rate of acid production. In such a case, the effect of ethanol on the rate of formaldehyde disappearance would be determined

Table 1. *Effect of alcohols on formaldehyde disappearance and acid production under the influence of liver enzyme*

(Manometric measurement of acid formation, with colorimetric estimation of residual formaldehyde at the end of the period of manometric observation. Enzyme concentration 1 mg./ml. Reaction time 65 min. Each flask contained initially 1 mg., i.e. 33·3 μmoles formaldehyde.)

	Methanol			Ethanol		
Alcohol concentration (M)	(*a*) Formaldehyde disappearance (μmoles)	(*b*) Acid formation (μmoles)	(*a*)/(*b*)	(*a*) Formaldehyde disappearance (μmoles)	(*b*) Acid formation (μmoles)	(*a*)/(*b*)
Nil	13·6	6·4	2·1	12·6	6·1	2·1
0·01	16·6	6·8	2·4	15·0	5·0	3·0
0·05	21·6	6·6	3·3	16·0	3·4	4·7
0·25	27·6	6·3	4·4	13·0	2·2	5·8

by the magnitudes of the reaction-velocity constants involved. If, in the simple formaldehyde-enzyme-Co I system, reaction (1) were the essentially slower reaction limiting the overall rate, its replacement by reaction (2) might increase the rate of formaldehyde disappearance. With methanol, on the other hand, the reaction corresponding to (3) is simply (2); no new reaction possibility would appear to have been introduced, and the rate of formaldehyde disappearance, if it is affected at all, should only be diminished.

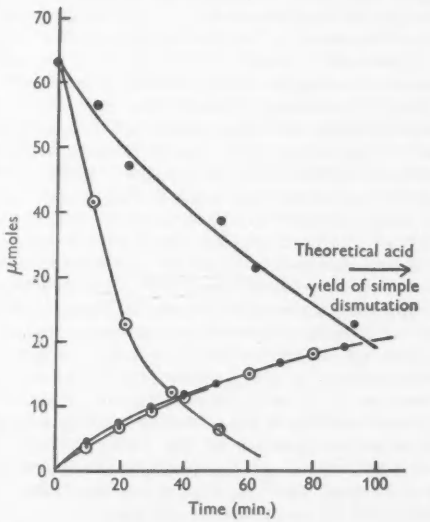


Fig. 2. Acid production (ascending curves) and formaldehyde content of the medium (descending curves) during the reaction of formaldehyde alone (●—●) and formaldehyde plus 0·25 M-methanol (⊙—⊙). Concentration of enzyme preparation, 1 mg./ml.

In further experiments the formaldehyde remaining in the medium after a period of manometric observation of acid formation was estimated. It became clear that the suggestion made above was quite inadequate to explain the results. Table 1 shows the formaldehyde disappearance and acid formation from formaldehyde alone, and from formaldehyde plus methanol or ethanol, under comparable conditions. In the absence of alcohol the molar ratio of formaldehyde disappearance to acid formation is not significantly different from the value of 2·0 expected for a simple mutase reaction. The presence of either alcohol in 0·01 M concentration has raised this ratio above the theoretical value, and at 0·25 M has more than doubled it. But the reason for the change is quite different in the two cases. With ethanol, it results from a depression of acid formation, with little change in formaldehyde disappearance. With methanol, there is no significant change in acid formation, but greatly increased formaldehyde disappearance. A possible explanation of the ethanol effect has already been outlined. Since the effect of methanol brought to light by the formaldehyde estimations fell outside the scope of that explanation, attention was now concentrated upon it.

Fig. 2 indicates the time course of the reaction at a particular enzyme concentration in systems containing formaldehyde alone and (formaldehyde plus methanol). The striking effect of methanol in accelerating formaldehyde disappearance is well shown, whilst the effect on acid formation is negligible. It is instructive to consider the magnitude of the ratio of formaldehyde disappearance to acid formation at different times during the reaction, and the change of this ratio with time is shown in Fig. 3. In the absence of methanol, the ratio remains constant near the value of 2·0 expected in a simple mutase reaction. In the presence of methanol, the ratio is more than 6 in the earliest stages; but it progressively falls, and if the incubation is sufficiently prolonged it, too, approaches a value of 2·0. The conclusion was unavoidable that a large fraction of the acid which ultimately appeared after prolonged incubation must have originated not

directly from formaldehyde, but by the rather slow degradation of some non-acidic intermediate product. The probable nature of this intermediate suggested itself when quantitative estimation of formate accumulating in the media had been carried out.

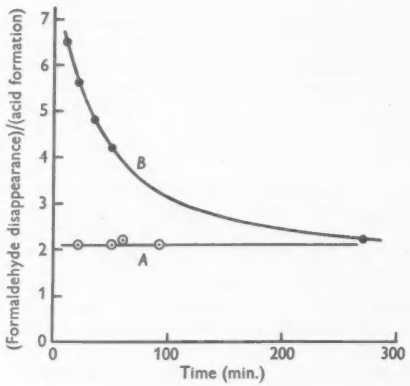


Fig. 3. Variation with time of the mol. ratio (formaldehyde disappearance)/(acid formation) during the reaction of *A*, formaldehyde alone; and *B*, formaldehyde plus methanol (data of Fig. 2).

Discrepancy between acid formation (manometric estimation) and formate production (chemical estimation). When data of the kind referred to in the preceding section were supplemented by chemical estimation of the formate present in the media at the end of the manometric period, it was found that the inclusion of the alcohols in the system resulted in a wide discrepancy between acid formation and formate production. The results set out in Table 2 are typical. As in the earlier experiments, acid production in the presence of either alcohol was much too small to accord with a simple dismutation of the aldehyde which had disappeared. In contrast, the chemical estimation of formate in the formaldehyde plus methanol system gave a figure greatly in excess of the acid formation, and a ratio of formaldehyde disappearance to formate production which could not be regarded as significantly different from the value of 2·0 expected in a simple dismutation. With formaldehyde plus ethanol, although the formate estimated chemically was greater than the acid production, it still fell far short of the requirement for dismutation of the formaldehyde disappearing.

These results made it necessary to postulate a conversion of formaldehyde into some non-acid substance which was nevertheless estimated quantitatively as formate by the Bastrup (1947) method. The first stage of the Bastrup procedure is a distillation of the sample with methanol and hydrochloric acid under conditions such that formic acid in the sample is converted into methyl formate, and passes over into an alkali trap. Such a procedure will clearly not distinguish between formate ions and volatile formate esters, and this consideration led to the formulation of a hypothesis that, in the presence of methanol, a considerable fraction of the oxidized product in the dismutation of formaldehyde was appearing as methyl formate and not as formic acid.

Appearance of volatile formate during the dismutation of formaldehyde in the presence of methanol or ethanol. The boiling points of the methyl and ethyl esters of formic acid are 31·5 and 54·3° respectively, and it was found that methyl formate in 0·1 M aqueous solution could be completely removed by aeration at room temperature in less than 30 min. As Bastrup (1947) has shown, methyl formate in such an air stream can be quantitatively trapped, with saponification, in sodium hydroxide solution and then determined according to his directions. It appeared, therefore, that aeration through media in which methyl formate accumulation was suspected into a suitable alkali trap might permit the demonstration of the presence of volatile formate. Reaction mixtures buffered with carbon dioxide-sodium bicarbonate were clearly unsuitable for such experiments, because of the necessity then of aerating with a gas mixture containing carbon dioxide which would rapidly exhaust the alkali trap. The following procedure was adopted.

Table 2. *Effect of alcohols on formaldehyde disappearance, acid formation and formate production under the influence of liver enzyme*

(Estimation of formate and residual formaldehyde after a period of manometric measurement of acid production. Enzyme concentration 1 mg./ml.; alcohol, when present, 0·25 M. Reaction time, 70 min. The figures refer to a volume of 6 ml., obtained by pooling the reaction mixtures from manometers run in triplicate in each series. The amount of formaldehyde initially present in this volume, corrected for a presumed 5% loss during gassing, was 95 μmoles.)

	Formaldehyde alone	Formaldehyde + methanol	Formaldehyde + ethanol
Acid formation (μmoles)	20·2	19·6	6·9
Formate production (μmoles)	23·8	36·2	12·4
Formaldehyde disappearance (μmoles)	53·3	81·7	61·7
Ratio: $\frac{\text{formaldehyde disappearance}}{\text{formate production}}$	2·2	2·3	5·0

The reaction vessel was a beaker fitted with a rubber stopper carrying gas inlet and outlet tubes, glass electrode and KCl-agar bridge, inlet tube for introduction of substrate and burette containing 0·01 N-NaOH. Enzyme and co-enzyme solutions, and alcohol if desired, in an appropriate volume of water, were placed in the beaker and the stopper was fitted to it. A current of CO_2-free N_2 was drawn through this, and passed thence through two absorption tubes in series, each containing 10 ml. 0·2 N-NaOH. With a slow gas stream stirring the medium, 0·01 N-NaOH was added from the burette until the pH was 7·4, as indicated by the glass electrode. The formaldehyde was added and the passage of gas increased to the limit imposed by the tendency to frothing. The latter was minimized by a smear of silicone (of unknown source) applied to the glass wall of the reaction vessel just above the fluid surface. During the reaction the pH was kept constant at 7·4 by addition of 0·01 N-NaOH from the burette as required, so that the amount added gave a measure of acid production. At the end of the experiment formate was estimated in the absorption tubes (the second of these never containing more than traces), as well as formate and formaldehyde remaining in the reaction medium. Controls showed that in the absence of enzyme no formate appeared in the traps.

The average results of two experiments are summarized in Table 3. Acid production was in reasonable agreement with the amounts of formate still present in the reaction media at the end of the experiment. The significant feature is the very large fraction (about 85 %) of the total formate production which was volatile at pH 7·4 and therefore carried over into the alkali traps, when methanol or ethanol was present. The more striking effect of methanol in accelerating formaldehyde disappearance is again manifest. The ratio of formaldehyde disappearance to total formate production is very near the value of 2·0, appropriate to a simple dismutation, in the formaldehyde alone and formaldehyde plus methanol systems, but in the formaldehyde plus ethanol system is significantly greater than this.

Effect of iodoacetate (IAA). Dixon & Lutwak-Mann (1937) found that their aldehyde mutase of liver was completely inactivated in a few minutes by 0·01 M-IAA, but Lutwak-Mann (1938) reported that liver alcohol dehydrogenase was scarcely affected by IAA at this concentration. This suggested that a study of the effect of IAA on the systems with which this paper is concerned might throw some light upon the reaction mechanisms involved. Fig. 4 shows the course of formaldehyde disappearance from reaction mixtures containing formaldehyde alone and

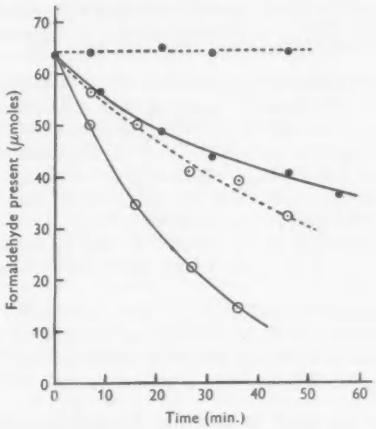


Fig. 4. Effect of iodoacetate on disappearance of formaldehyde. Bicarbonate buffer, pH 7·4, at 37°. Enzyme concentration, 2 mg./ml. ●, formaldehyde alone; ⊙, formaldehyde plus 0·25 M-methanol. Continuous lines, without IAA; broken lines, with 0·01 M-IAA.

(formaldehyde plus methanol), in each case both with and without 0·01 M-IAA. When formaldehyde alone was the substrate, inhibition by IAA was as complete as Dixon & Lutwak-Mann had found it to be. In the presence of methanol, on the other hand, the effectiveness of the IAA inhibition was less than 50 %, and the rate of formaldehyde disappearance in 0·01 M-IAA was greater than when formaldehyde alone was the substrate, without IAA. The effect of IAA on the removal of formaldehyde was found to be least of all in the presence of ethanol (Table 4).

Table 3. *Appearance of volatile formate during the dismutation reaction*

(Volume of reaction mixture 20 ml., containing enzyme preparation (2 mg./ml.), formaldehyde (1 mg./ml.), alcohol, when present (0·25 M). Temp., 22°; pH maintained constant at 7·4. Vigorous bubbling of N_2 during the reaction, passing gas into absorption tubes containing 0·2 N-NaOH. Reaction time, 2 hr. The quantities, in μmoles, are the average of closely agreeing duplicates.)

	Formaldehyde alone	Formaldehyde + methanol	Formaldehyde + ethanol
Alkali required to keep pH at 7·4	47	29	15
Formate found in reaction medium	58	35	19
Formate found in absorption tubes	42	249	102
Total formate production	100	284	121
Formaldehyde disappearance	194	551	213
Ratio: $\frac{\text{formaldehyde disappearance}}{\text{formate production}}$	1·9	1·9	2·6

Table 4. *Effect of iodoacetate on formaldehyde disappearance*

(Bicarbonate buffer at pH 7·4 and 37°, for 34 min. Enzyme preparation (2 mg./ml.), alcohol, when present (0·25 M). The figures refer to a reaction mixture volume of 2 ml. which contained initially 63 μmoles formaldehyde. Averages of closely agreeing duplicates or triplicates.)

	Formaldehyde alone		Formaldehyde + methanol		Formaldehyde + ethanol	
IAA (0·01 M)	−	+	−	+	−	+
Formaldehyde disappearance (μmoles)	20·4	1·2	46·8	23·1	23·1	18·7

The persistent disappearance of formaldehyde in the alcohol-containing media, in spite of the presence of a concentration of IAA sufficient completely to inhibit simple mutase activity in the absence of alcohol, raised the question as to whether volatile-formate production and the acceleration by alcohol of formaldehyde disappearance, were not solely determined by the activity of the alcohol dehydrogenase in the enzyme preparation (see Discussion section).

Colorimetric evidence of ester formation. The application of the hydroxamate method of ester determination finally proved to be the simplest way of demonstrating ester accumulation in reaction mixtures. This method has so far been used only in a small number of experiments, but some results which give an indication of the relationship between alcohol concentration and ester formation are shown in Table 5. The accumulation of ester appears to be maximal when the alcohol concentration is about 0·1 M and, in the case of ethanol, higher concentrations have an inhibitory effect. This last may be due to the operation of reaction (3) as suggested earlier. Ester formation occurred to an appreciable extent in 0·01 M-alcohol. Small amounts of ester are recorded as finally present when the media contained initially no alcohol; but such quantities are near the limit of accuracy of the analytical method and cannot at present be accepted as significant. Even in the absence of added alcohol, some ester formation might occur in the later stages of the reaction, through the influence of methanol formed by the dismutation.

Table 5. *Ester formation in formaldehyde-alcohol mixtures at various alcohol concentrations*

(0·1 M-Na_2HPO_4-KH_2PO_4 buffer pH 7·4 and 22°. Enzyme preparation (0·8 mg./ml.), formaldehyde (0·033 M). Ester determined by the hydroxamate colorimetric method in 2 ml. of reaction mixture after a reaction period of 1 hr.)

Alcohol concentration (M)	Ester accumulation (μmoles) in presence of	
	Methanol	Ethanol
Nil	0·5	0·5
0·01	1·8	1·5
0·10	6·0	2·1
1·00	5·7	0·9

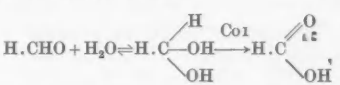
Stability of methyl formate in the reaction mixtures. The largest yields of ester were obtained in experiments at room temperature in which the ester was removed from the medium quite rapidly after its formation, by a stream of gas. In the manometric experiments at 37°, with prolonged incubation of reaction mixtures in which formaldehyde plus methanol was the substrate, ester accumulating during the early stages was subsequently hydrolysed. In the experiment to which Figs. 2 and 3 refer, about 95% of the formaldehyde had disappeared within 1 hr., but the ratio of formaldehyde disappearance to acid formation was then 4. After 4 hr. this ratio was reduced to a value very little greater than 2, indicating an almost complete hydrolysis of the ester which had been present. The stability of methyl formate in 0·1 M-Na_2HPO_4-KH_2PO_4 buffer (pH 7·4) and at room temperature was tested, using the hydroxamate method for the estimation of unhydrolysed ester. The plot of the logarithm of ester concentration against time under these conditions was linear, and the half-life of the ester was about 5 hr. The inclusion in the system of 1 mg./ml. of enzyme preparation accelerated the hydrolysis and reduced the half-life to about 1·5 hr., presumably as a result of the presence of esterase in the liver enzyme preparation. Similar tests at 37° and in bicarbonate buffer have not been carried out, because of difficulties due to the low boiling point of methyl formate, but the results obtained at room temperature make it probable that the rate of hydrolysis of the ester in the presence of the enzyme preparation has an order of magnitude consistent with our interpretation of events in the formaldehyde-methanol system.

DISCUSSION

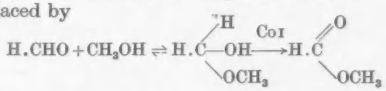
We believe that the experimental results which have been described provide ample proof of the accumulation of formic ester as a primary product of the enzymic dismutation of formaldehyde in the presence of methanol or ethanol. In the simpler case, in which the substrate is formaldehyde plus methanol, the evidence for methyl formate production may be summarized as follows: (*a*) formate, estimated chemically by a method which does not distinguish between formic ester and free formate,

is found to be produced in the proportion of 1 mol. for every 2 mol. of formaldehyde which have disappeared, which is in accord with simple dismutation theory. (*b*) Total acid production, estimated manometrically, is very much smaller than total formate production during the early stages of the reaction, but the difference becomes less as a result of prolonged incubation. (*c*) A large fraction of the formate appearing is sufficiently volatile from solution at pH 7·4 to be rapidly removed by a stream of gas at room temperature. (*d*) The application of the colorimetric hydroxamate method of ester determination gives confirmation of the presence of ester in the reaction mixtures.

While Dixon & Lutwak-Mann (1937) inclined to the view that the aldehyde mutase of liver was an enzyme distinct from the alcohol and aldehyde dehydrogenases, more recent developments, and in particular the isolations from liver of aldehyde dehydrogenase by Racker (1948) and crystalline alcohol dehydrogenase by Bonnichsen & Wassen (1948), have increased the probability that mutase is a coenzyme-linked system with aldehyde and alcohol dehydrogenase components. It is widely believed that it is the hydrated form of an aldehyde molecule which undergoes dehydrogenation to give the corresponding carboxylic acid, and in their preliminary communication Kendal & Ramanathan (1951) suggested that the appearance of methyl formate instead of formic acid in the formaldehyde plus methanol systems might be due to the presence of a sufficient amount of semi-acetal under these conditions for this to compete effectively with the hydrated form as a substrate for aldehyde dehydrogenase. The reactions



giving formic acid as the end product would then be replaced by



with methyl formate as the end product. The further supposition, that under the experimental conditions the rate of the second reaction was faster than the first, was necessary to account for the very pronounced accelerating effect of methanol on the overall rate of formaldehyde disappearance.

The results of the experiments with IAA raised a doubt as to the identity of the enzyme responsible for methyl formate production. Reference has already been made to the findings of Dixon & Lutwak-Mann (1937) that liver aldehyde mutase is completely inactivated by 0·01 M-IAA, and of Lutwak-Mann (1938) that liver alcohol dehydrogenase is not affected by IAA in this concentration. This suggests that it is only the aldehyde dehydrogenase component of the mutase which is so sensitive to IAA. In our experiments, the disappearance of formaldehyde from the simple enzyme-coenzyme-formaldehyde system was almost completely prevented by the addition of IAA, but when methanol or ethanol were also present, formaldehyde disappeared at a considerable rate in spite of the IAA. The rate in the case of the methanol system was in fact about the same as the increase in rate caused by the presence of methanol in the absence of IAA. These new facts make it likely that it is the alcohol dehydrogenase and not the aldehyde dehydrogenase which is able to dehydrogenate the semi-acetal. And indeed it may be argued that structural considerations favour this revision of opinion. The similarity between the semi-acetal $CH_3O.CH_2OH$ and the substrate *par excellence* of alcohol dehydrogenase, $CH_3.CH_2OH$, would seem to be much closer than that between the first of these molecules and the substrate $HO.CH_2OH$ of the aldehyde dehydrogenase. A rather surprising feature of the earlier experiments was the absence of any significant effect of methanol on the acid production in the early stages of the reaction, in spite of its very marked effect on formaldehyde disappearance. In terms of semi-acetal and substrate competition for the aldehyde dehydrogenase, it did not seem very likely that a diminution in direct formic acid production as a result of the substrate competition would be so exactly balanced by formic acid produced indirectly by hydrolysis of methyl formate. This difficulty is largely resolved when the semi acetal is postulated as a substrate of the alcohol dehydrogenase only; the direct formation of formic acid by the aldehyde dehydrogenase at the beginning of the reaction may then proceed uninfluenced by the presence of methanol. Conclusive proof of the postulate that liver alcohol dehydrogenase alone, in the presence of coenzyme and methanol, brings about a 'dismutation' of formaldehyde to methanol and methyl formate awaits the results of further work with crystalline alcohol dehydrogenase devoid of aldehyde dehydrogenase activity.

One may conclude from the observations made when ethanol was present with the formaldehyde that ethyl formate is then formed, but on a smaller scale. Total formate never accounted for half the formaldehyde which disappeared, and it is presumed that in this case a significant fraction of the oxidation of $Co_I H_2$ by formaldehyde to give methanol was balanced by a reduction of Co_I by ethanol to give $Co_I H_2$ and acetaldehyde. This is the more likely because of the much higher affinity of ethanol, compared with methanol, for alcohol dehydrogenase.

It must be admitted that there is at present no justification for an assertion that this new mechanism of ester formation is of real biochemical significance, but the phenomenon is of interest as an instance of enzymic ester formation which does not depend on the synthetic activity of an esterase. It is perhaps the kind of mechanism which might be responsible for the appearance of ethyl acetate in growing cultures of those yeasts which, as Peel (1951) has shown, produce this ester in a concentration greater than can be explained in terms of esterase-catalysed synthesis from ethanol and acetic acid. One is tempted also to speculate whether methyl formate might be a factor in determining the specific toxic effects of methanol. There seems to be fairly general agreement among students of methanol poisoning that the specific toxic effects (e.g. those on the retina) are not due to methanol itself, but to its metabolic products. They have been variously attributed to the localized action of formaldehyde or formic acid, but it has not been possible to reproduce them satisfactorily in experimental animals by the administration of these substances. Methanol, like ethanol, distributes itself rapidly and uniformly throughout the body water after it has been ingested. A dose of only 5 g., in man, produces a peak concentration of about 0·1 mg./ml., i.e. 0·003M (Leaf & Zatman, 1952). Dangerous doses, from about 10 g. upwards, may consequently be expected to give rise to concentrations which might, after primary conversion of part of the methanol into formaldehyde, lead to a secondary conversion of the latter into methyl formate by a semi-acetal dehydrogenase mechanism. Furthermore, the preferential fat-solubility of the ester is a property which might result in localization and specific effects.

SUMMARY

1. The ability of certain liver enzyme preparations to bring about the dismutation of formaldehyde has been confirmed.

2. When methanol was present in the system, the disappearance of formaldehyde was greatly accelerated, but without any corresponding acceleration of acid production. The appearance of formate was, however, increased to a degree corresponding with the acceleration of formaldehyde utilization.

3. After prolonged incubation of the reaction media, acid formation continued to increase and finally became equal to formate production.

4. The discrepancy between acid and formate production during the early stages of the reaction was due to the accumulation of a volatile ester of formic acid. The ester subsequently underwent a slow hydrolysis.

5. It is suggested that the ester formed from formaldehyde in the presence of methanol is methyl formate, and that it arises as the product of dehydrogenation of the semi-acetal formed from formaldehyde and methanol.

6. Volatile-ester formation occurs also in an enzyme-formaldehyde-ethanol system, but to a smaller extent.

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Metabolic Studies with 3-Methyl Glucose

1. ITS FATE IN THE ANIMAL BODY

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The balance of available evidence suggests that in glycogen and starch the hydroxyl groups in position 3 of the glucose residues are free. It therefore was possible that glucose carrying a substituent at position 3 might, in the animal body or elsewhere, be converted to a methyl derivative of glycogen. Many years ago unpublished preliminary experiments by one of us (F.G.Y.) indicated that 3-methyl glucose was not appreciably converted to glycogen in the liver or the muscles of the rabbit. This failure of the animal to utilize 3-methyl glucose to an appreciable degree, although it is a simple sugar derivative which closely resembles glucose in many chemical and physical properties, has been further investigated. A preliminary account of the results obtained has been recorded (Campbell, 1949).

The present paper describes experiments with the intact animal, while the following one (Campbell, 1952) is concerned with investigations *in vitro*.

EXPERIMENTAL

Synthesis, properties and estimation of 3-methyl D-*glucopyranose*

The synthesis from D-glucose followed that generally employed (Irvine & Scott, 1913; Levene & Meyer, 1922) in which D-glucose is converted to 1:2–5:6-di*iso*propylidene-D-glucofuranose, the only free OH group of which is then methylated and the *iso*propylidene groups removed by acid hydrolysis. After neutralization of the solution and evaporation to dryness the 3-methyl glucose is obtained as a white crystalline material by crystallization from methanol containing a little acetone. Di*iso*propylidene-glucose was prepared by the method of Bell (1935), although the procedure devised by Raymond & Levene (1929) for the separation of the di*iso*propylidene from the monopropylidene derivative, in which the former is extracted with boiling light petroleum (80–100°), was found to be a useful simplification of the method. From 100 g. of D-glucose about 25 g. of 3-methyl glucose were obtained.

An aqueous solution of the sugar had $[\alpha]_D^{20°}$ of $+98{\cdot}0° \pm 0{\cdot}1$ falling to $+58{\cdot}5° \pm 0{\cdot}1$. The corrected melting point was 157·5°. With Benedict's (1926) quantitative copper reagent the reducing power was 45% of that of glucose and approximately 80% with the Hagedorn & Jensen (1923*a*, *b*) $K_3Fe(CN)_6$ reagent. 3-Methyl glucose forms an osazone which has a characteristic crystalline formation which, although similar to that of glucosazone, can be differentiated from the latter under the microscope. A solution of 3-methyl glucose is not fermented by ordinary baker's yeast.

The content of 3-methyl glucose in pure solution and in tissue fluids containing the sugar was determined in two ways: (*a*) by the method of Zeisel (1885), with the volumetric procedure due to Viebök & Brecher (1930), in which the methoxyl content of the solution is determined; the procedure adopted followed that described by Elek (1939) with only minor modifications; (*b*) by removal of the glucose by means of yeast fermentation, and estimation of the residual reducing power of the solution. This method is based on that of Young (1934) and for plasma is as follows: to 2 ml. $ZnSO_4$ solution (1·8%) is added 1 ml. plasma followed by 2 ml. 0·085 N-NaOH. After 3 min. in a boiling-water bath the precipitate is filtered into a 30 ml. beaker and washed with 3 × 2 ml. hot distilled water. The beaker is placed in an oven at 37° for 15 min., 2 ml. yeast suspension, prepared according to the method of Somogyi (1927) at room temperature, are added and the whole incubated at 28° for 30 min. During this time the beakers are covered with watch glasses and are occasionally stirred. The yeast is then filtered off on a sintered-glass funnel (Jena 4G) and washed three times with 2 ml. distilled water. The filtrate is made up to 25 ml. with distilled water and suitable samples are taken for Hagedorn & Jensen estimations. The reducing value of the yeast suspension and the non-sugar reducing substances in the plasma is allowed for by a control estimation on a sample of plasma which does not contain 3-methyl glucose.

The fate of 3-methyl glucose fed to the rat

The methods adopted followed closely those previously used by Cori (1926). The rats were arranged in pairs so that the total weight of each pair was approximately the same. The rats were starved and the urines collected for the 24 hr. period prior to the experiment. The rats in each group then received by stomach tube either 2·5 ml. of 50% (w/v) glucose or 3-methyl glucose, or 2·5 ml. water. The solutions were warmed to 37° before administration. The rats were immediately replaced in their metabolism cages in pairs and the urine collection started afresh. After a 4 hr. absorption period each rat was killed by an intraperitoneal injection of 0·5 ml. of Nembutal. As soon as the animal had lost consciousness the head was removed and as much blood collected as possible.

The liver was next removed, wiped free from blood on a filter paper, weighed and immediately placed in a boiling tube with 5 ml. of hot 60% (w/v) KOH. The bladder was

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emptied and the contents added to the sample of urine already obtained. The gut was ligatured at both ends, carefully dissected away, and placed in a beaker of distilled water. The body was completely eviscerated and skinned and the carcass placed in a beaker containing 60 ml. hot 60% KOH. Estimations were then carried out as follows. The liver and carcass glycogen content was determined according to the method of Evans, Tsai & Young (1931). The methoxyl content of 4 ml. samples of the acid hydrolysates of the precipitated glycogens was determined by the Zeisel method. The estimations of the latter were carried out in pairs, one estimation on a sample of an acid hydrolysate from a rat fed 3-methyl glucose and the other on a sample from a rat fed either glucose or water. Each pair of estimations was carried out on the same day with the same sample of HI.

As a check on the methoxyl content of the glycogens, some pure glycogen was isolated from the original alkaline hydrolysates of the carcasses in order to estimate the methoxyl content of the unhydrolysed glycogen samples. The method used for the isolation of the glycogen samples was that described by Somogyi (1934), their methoxyl content being estimated by the Zeisel method.

The extent of the absorption of the sugars from the intestine was determined by the method described by Cori (1926). The reducing power of the gut contents was estimated by the method of Hagedorn & Jensen. The amount of sugar fed to each rat was determined by passing an identical quantity of the sugar solution through the same catheter as that used in the rats into a volumetric flask, after which the volume was made up with distilled water.

Two 0·1 ml. samples of blood were taken from each rat and the blood sugar concentrations determined by the usual Hagedorn & Jensen technique. The rest of the blood was used to determine the concentration of 3-methyl glucose by the Zeisel method. The blood collected from each rat was added to a 25 ml. measuring cylinder containing 10 ml. 10% (w/v) trichloroacetic acid. After the blood had been well mixed with the acid the precipitate was removed on a dry filter paper and 4 ml. samples of filtrate were used for the methoxyl estimations. The filtrate from the blood of the glucose-fed rats served for a control estimation.

Benedict's qualitative test for reducing sugars was carried out on the urines from each of the three groups of rats. If sugar was present the amount was estimated by Benedict's quantitative method, and in the case of the 3-methyl glucose-fed rats the methoxyl content of the urines was also estimated by the Zeisel method. Since urinary sulphate interferes with the Zeisel estimation this had to be removed by treatment of the urine with hot 2N-HCl, followed by precipitation of the sulphate as $BaSO_4$. The filtrate was then used for the estimations. Control experiments showed that no significant amount of 3-methyl glucose was lost during this process.

The nature of the substances excreted after the administration of 3-methyl glucose and the recovery of the sugar in the urine

The volatile reducing substances in the urines were estimated by a method based on that described by Archer, Chapman, Rhoden & Warren (1948) for the determination of the volatile reducing substances in blood. Each determination was made on 2 ml. urine to which approx. 8 ml. water were added. Trouble due to frothing was overcome by the addition of a little glass wool to the distilling flask. The presence of glucose in the urine of rats fed 3-methyl glucose was tested by fermentation of a sample of the urine with baker's yeast at 28°.

Osazones were prepared from the urine of rats which had received 2 ml. of a 6% solution of 3-methyl glucose by intraperitoneal injection, and from the urine of normal rats to which a quantity of 3-methyl glucose had been added so that the methoxyl content of each urine was similar. Glucosazone was prepared from another urine to which sufficient glucose had been added to bring the concentration of sugar to approximately that of the experimental urines. The crystal structures of the osazones from the three groups of urines were compared under the microscope.

Two series of experiments were carried out in order to determine the extent to which the administered 3-methyl glucose could be recovered from the urine. In the first, the rats were given the sugar or water by mouth, as described above, and the urine collected by means of a separator as described by Maw (1948). The volume of urine from each group of rats was measured 4, 8 and 24 hr. after feeding, and the presence of reducing sugar tested with the Benedict quantitative reagent. The urine collection from the rats fed glucose and water was stopped after 24 hr., but the collection from the rats fed 3-methyl glucose was continued for several hours after the time at which the presence of reducing sugar could no longer be detected. Zeisel methoxyl estimations were then carried out on the urines. The rats were kept under observation for several weeks as it was of interest to know what long-term effect, if any, the treatment had.

In the second series of experiments the recovery of 3-methyl glucose was studied after an intraperitoneal injection of the sugar. Two pairs of rats, the total weight of each pair being approximately the same, were starved overnight. One pair was then given 2 ml. 3-methyl glucose (6%) solution and the other pair a similar quantity of 0·9% saline, by intraperitoneal injection. The urines were collected over the following 48 hr. during which time the rats were given a diet of cellulose. Urine was then collected from the experimental pair for a further 2 hr. and if this specimen failed to reduce Benedict reagent the collection of urine was stopped. The volume of urine excreted by each pair of rats during the 48 hr. period was recorded and the volume of the smaller sample brought to that of the larger by the addition of distilled water. The control urine was divided into halves. The same quantity of the same 3-methyl glucose solution as was given to one rat was then added to one half, and a similar quantity of 0·9% saline added to the other half. Zeisel methoxyl and Benedict estimations were then made on all the samples. These rats were also kept under observation for several weeks.

RESULTS

Glycogen content of liver and carcass

The livers of the rats given 3-methyl glucose by stomach tube possessed less than one-quarter, and those of the control rats given water less than one-ninth, of the glycogen content of those of the glucose-fed animals (Table 1). The differences between the mean results for the glucose-fed animals and

Table 1. *Glycogen content of livers and carcasses of rats given glucose or 3-methyl glucose by stomach tube*

(Results represent the mean for six animals. The glycogen content is expressed as equivalent glucose.)

	Glycogen content (g./100 g. fresh tissue) of animals given			Probability of difference being due to chance alone		
Source of glycogen	(*a*) Glucose	(*b*) 3-Methyl glucose	(*c*) Water	*a* and *b*	*a* and *c*	*b* and *c*
Liver	1·99±0·25	0·44±0·17	0·20±0·07	<0·001	<0·001	0·20–0·30
Carcass	0·26±0·031	0·12±0·012	0·11±0·008	0·001–0·01	<0·001	0·40–0·50

Table 2. *Methoxyl content of the glycogen from the liver and carcass of rats fed glucose and 3-methyl glucose, and of pure glycogen from each group*

	'Apparent % OMe' in glycogen in animals given		Probability of difference being due to chance alone
Source of glycogen	(*a*) Glucose or water	(*b*) 3-Methyl glucose	*a* and *b*
Liver*	1·17±0·70	1·42±0·29	0·50–0·60
Carcass*	2·99±0·48	2·69±0·33	0·60–0·70
Pure glycogen from carcass	0·02 0·11	0·30	—

* Mean results for estimations on six different samples.

Table 3. *Concentration of sugar in the blood of rats given glucose or 3-methyl glucose by stomach tube*

	Concentration (mg./100 ml.) of sugar in the blood of animals given				
	Glucose	3-Methyl glucose			Water
Exp. no.	Total reducing power (as glucose)	Total reducing power (as glucose)	3-Methyl glucose (from Zeisel)	Glucose (by difference)	Total reducing power (as glucose)
1	132	184	75	124	109
	130	176	80	112	104
2	134	246	187	123	100
	141	225	156	119	118
3	124	223	180	106	100
	135	232	189	110	93

those for the other groups are highly significant statistically, while the difference between the mean results for the rats given 3-methyl glucose and those given water is not statistically significant, on the number of animals in these experiments, at a 5% level of significance.

The carcasses of the rats given 3-methyl glucose and of those given water possess similar contents of glycogen (Table 1), amounting to about one-half of the glycogen content for the rats fed glucose. The mean figures for these two groups are not significantly different statistically. The differences between the mean figures for the glucose-fed rats and those for the other two groups are highly significant.

When acid hydrolysates of the glycogen from the livers and carcasses of the sugar-fed rats are examined for the presence of methoxyl groups (Table 2) no significant difference is found between the results for the animals given glucose and for those given 3-methyl glucose. The apparent presence of methoxyl groups in these crude hydrolysates is not to be attributed to the presence of methoxyl groups in the glycogen itself since the glycogen isolated from these tissues gave virtually negative methoxyl results (Table 2). The magnitude of these data for 'apparent % OMe' is to be attributed to the fact that blank experiments could not be carried out simultaneously when hydrolysates from glucose- or water-fed rats, and from 3-methyl glucose-fed rats were being simultaneously estimated. It was of course a comparison of the data for the two groups which was of importance, and not the absolute values.

Concentration of sugar in the blood of the sugar-fed animals

The blood-sugar level (calculated as glucose) of the 3-methyl glucose-fed rats is very much higher than the corresponding level for the rats fed either glucose or water (Table 3). Owing to the fact that the reducing power of 3-methyl glucose is less than that of glucose the real blood sugar level, as opposed to that referred to glucose, in the case of the 3-methyl glucose-fed rats is rather higher than that given in the table.

The concentration of 3-methyl glucose in the blood of the 3-methyl glucose-fed rats was determined from the Zeisel estimations and the approxi-

mate blood glucose concentration in these rats was calculated by difference. It will be seen that the concentration of glucose in the blood of the 3-methyl glucose-fed rats is substantially the same as that of the water-fed rats (Table 3).

Absorption of sugar from the gut

No significant amount of sugar was found in the gut of the glucose-fed rats, whereas in two experiments 100 and 180 mg. respectively of reducing sugar (calculated as 3-methyl glucose) were found in the filtrates of the gut contents of the 3-methyl glucose-fed rats. Since about 1 g. of sugar was fed to each rat, about 15% of the administered 3-methyl glucose was unabsorbed after the 4 hr. absorption period.

The nature of the substances excreted after the administration of 3-methyl glucose

The reduction tests on the urines excreted during the 4 hr. absorption period after feeding the rats by stomach tube showed that only the urines excreted by the 3-methyl glucose-fed rats reduced the Benedict qualitative reagent. Zeisel methoxyl and Benedict quantitative estimations were carried out on all these urines, using the urine excreted by the same rats during the previous 24 hr. as controls in the methoxyl estimations. Table 4 gives the concentration of sugar in the urines, assuming the latter to be 3-methyl glucose, as determined by the two methods, and the total amount of sugar excreted during the absorption period. The close agreement between the results obtained suggests that the sugar in the urine was probably 3-methyl glucose. Since about 2 g. of sugar were fed to the pair of rats about 25% of the fed sugar was excreted during the 4 hr. absorption period. It will also be observed that the concentration of the sugar in the urine is about 6%, which is approximately the strength of a solution of a hexose isotonic with the blood.

Table 4. *Estimation of 3-methyl glucose in the urine of rats given 3-methyl glucose by stomach tube*

Exp. no.	Concn. of 3-methyl glucose (mg./ml.) in the urine as estimated by: Benedict's method	Concn. of 3-methyl glucose (mg./ml.) in the urine as estimated by: Zeisel's methoxyl method	Total amount of 3-methyl glucose excreted during absorption period (Zeisel's method) (mg.)
1	65	71	750
2	46	47	550
3	68	62	550

Table 5 shows the titration readings obtained in the volatile reducing matter estimations with the urine of rats which had been fed 3-methyl glucose, glucose and water. There is no significant difference between the results obtained from the three groups and it is concluded, therefore, that there is no significant increase in the volatile reducing matter, e.g. methanol or formic acid, in the urine as a result of feeding 3-methyl glucose to a rat.

Table 5. *Estimation of volatile reducing matter in the urine of rats given glucose or 3-methyl glucose by stomach tube*

Urine sample	Concn. (ml. 0·1 N-$Na_2S_2O_3$/ml. of urine) of volatile reducing matter in the urine of rats given: Glucose	3-Methyl glucose	Water
24 hr. before feeding	0·84, 0·76	0·86, 0·81	0·86, 0·80
24 hr. after feeding	0·84, 0·80	0·82, 0·74, 0·79	0·74, 0·71

Fermentation studies on the urines showed that there was no abnormal amount of glucose present as a result of feeding 3-methyl glucose.

There was an obvious similarity in the crystal structure of the osazones prepared from the urines excreted by the rats which had been administered 3-methyl glucose and the urine to which 3-methyl glucose had been added. These crystals differed markedly from those of the osazones prepared from urine to which glucose had been added. Thus it is concluded that the urines from the rats which had received an intraperitoneal injection of 3-methyl glucose contained a considerable amount of the sugar.

Recovery of sugar in the urine

In the recovery experiments the urine excreted by the rats fed 3-methyl glucose reduced the Benedict qualitative reagent 4, 8, 24 and 28 hr. after feeding by stomach tube, but urine collected at 31 hr. failed to reduce the reagent. The amount of 3-methyl glucose excreted in the urine up to that time was determined. In two experiments recoveries of 71 and 80% were obtained. None of the rats used in these experiments showed any pathological symptoms while under observation.

Table 6. *Recovery of 3-methyl glucose in the urine of rats given 3-methyl glucose (6% solution) by intraperitoneal injection*

Exp. no.	Comparison of Zeisel methoxyl titres (ml. 0·01 N-$Na_2S_2O_3$/total urine volume) for the urines containing: Excreted 3-methyl glucose	Added 3-methyl glucose	Recovery of the injected sugar in the urine (%)
1	10·3	11·9	87
2	7·90	8·13	97

In the experiments in which 3-methyl glucose was given by intraperitoneal injection, no urine collected more than 48 hr. after the injection of the sugar reduced the Benedict qualitative reagent. The methoxyl determinations were, therefore, carried out on the urines excreted during this 48 hr. period. In this case a comparison of the titration readings from the Zeisel estimation for the control urine to which 3-methyl glucose had been added and the urine excreted by the 3-methyl glucose-fed rats provides the best measure of the recovery of the administered sugar. The results of the two experiments are summarized in Table 6. The average recovery in the two experiments was 92 %. Thus the recovery was rather higher when the sugar was administered by intraperitoneal injection than when it was fed by stomach tube. The reducing power of the excreted sugar was identical with 3-methyl glucose. Again no pathological symptoms developed in any of the rats kept under observation after the completion of the experiments.

The diuretic effect of 3-methyl glucose

The volume of urine excreted by the three groups of rats during the 4 hr. absorption period after feeding glucose, 3-methyl glucose and water by stomach tube was measured. Approximately twice as much urine was excreted by the six 3-methyl glucose-fed rats (30·9 ml.) during the period as by either of the other two groups of rats (12·0 and 14·7 ml.). In Table 7 the urine volumes in a similar experiment which was continued for a rather longer period are recorded. This shows that while 3-methyl glucose has a diuretic effect for about the first 8 hr. after feeding, over a 24 hr. period no diuretic effect is observable.

Table 7. *Volume of urine excreted by rats given glucose and 3-methyl glucose by stomach tube*

Period of excretion of urine	Volume of urine (ml.) excreted by pairs of animals given		
	Glucose	3-Methyl glucose	Water
24 hr. before feeding (av. for Exps. 1 and 2)	17	20	18
7 hr. after feeding (Exp. 2)	10	15	9·2
8 hr. after feeding (Exp. 1)	6·2	19	6·4
24 hr. after feeding (av. for Exps. 1 and 2)	17	20	18

DISCUSSION

The methoxyl determinations on the acid hydrolysates of the glycogen samples from the carcass and liver after feeding 3-methyl glucose showed that no significant amount of methyl glycogen is formed in the liver. A comparison between the carcass glycogen levels of the rats fed 3-methyl glucose, glucose and water suggests that 3-methyl glucose does not give rise to any significant amount of carcass glycogen. Although there is statistically no difference between the liver glycogen contents in the water- and 3-methyl glucose-fed rats the mean content for the latter group was twice that for the former. The possibility that 3-methyl glucose can be utilized to a very small extent cannot be excluded. Since Hurlock & Tosic (1951) have found a strain of micro-organism that will utilize 3-methyl glucose, it is perhaps not surprising if some small degree of utilization of methyl glucose in the animal body does indeed occur.

The observed rise in the concentration of sugar in the blood after feeding 3-methyl glucose would be expected with a sugar which is not being readily metabolized. The fact that the glucose concentration of the blood is not seriously affected suggests that 3-methyl glucose has little or no effect on the hormonal control of the blood-sugar level.

Our results concerning the rate of absorption of 3-methyl glucose from the gut are in general agreement with the view that this sugar is actively absorbed (Campbell & Davson, 1948; see also Csáky, 1942).

The experiments on the nature of the substances excreted after the administration of 3-methyl glucose show that the unchanged sugar is present in the urine. The fact that there was no significant increase in the concentration of the volatile reducing matter in the urine after feeding the sugar, suggests that there is no extensive breakdown of the sugar, for this would probably lead to the formation of either methanol or formic acid, either of which substances would have affected the content of volatile reducing substances in the urine.

A rather higher rate of recovery of the sugar in the urine might be expected when the sugar is fed by intraperitoneal injection, rather than by stomach tube, as in the former case the possibly complicating factor of intestinal absorption is absent. The average figure for the recovery from the urine (92 %) is rather low if indeed the sugar is not metabolized at all, although this low recovery may result from the experimental difficulties involved. Nevertheless, the possibility that a very small amount of the sugar is indeed metabolized cannot be excluded.

SUMMARY

1. The administration of a solution of 3-methyl glucose to normal rats did not lead to a significant increase in the glycogen content of the liver or carcass nor to the formation of any significant amount of monomethyl glycogen in the liver or carcass.

2. No significant increase in the amount of volatile reducing substances in the urine took place after the administration of 3-methyl glucose to the rat, but a reducing sugar shown to be unchanged 3-methyl glucose was excreted.

3. An average recovery in the urine of 92 % of the administered sugar was obtained after an intraperitoneal injection of 3-methyl glucose to the rat.

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Metabolic Studies with 3-Methyl Glucose

2. *IN VITRO* STUDIES

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From the experiments reported in the previous paper (Campbell & Young, 1952) it appears improbable that 3-methyl glucose is metabolized to any appreciable extent *in vivo* in the rat. It was, therefore, of interest to determine whether any evidence for its metabolism could be obtained from *in vitro* studies. First, the effect of 3-methyl glucose on the rate of fermentation of glucose by yeast was studied. Secondly, since Cori, Cori & Green (1943) had shown that glucose strongly inhibited the conversion of glucose-1-phosphate to polysaccharide with a crystalline muscle phosphorylase preparation it was of interest to know if 3-methyl glucose possessed similar properties. Finally, an attempt was made to synthesize 3-methyl glucose-1-phosphoric acid so that the suitability of this substance as a substrate for phosphorylase could be studied.

EXPERIMENTAL

Experiments with yeast

The effect of 3-methyl glucose on the rate of fermentation of glucose by yeast was studied by following the loss of reducing power of a reaction mixture with and without the addition of 3-methyl glucose during the fermentation of glucose by yeast. It was first necessary to determine the conditions under which the loss of reducing sugar was linearly related to time. The fermentations were carried out in a similar manner to that already described for the estimation of 3-methyl glucose in plasma (Campbell & Young, 1952), using 2 ml. of yeast suspension, 2 ml. of 5 % (w/v) glucose solution, and 2 ml. of 5 % 3-methyl glucose solution or water. The fermentation was stopped at 0, 10, 20 and 30 min. respectively by the addition of 2 ml. of a 5 % solution of $ZnSO_4$ and 2 ml. of saturated $Ba(OH)_2$ solution, quickly followed by removal of the yeast by filtration. All fermentations were carried out at the same time so that the conditions were as nearly as possible the same in all cases. The reducing power of a 3-methyl glucose solution before and after incubation with yeast was determined as a control experiment.

Experiments with a muscle phosphorylase preparation

The experimental procedure was based on that of Cori *et al.* (1943) in which the synthesis of a polysaccharide from glucose-1-phosphate with crystalline muscle phosphorylase is followed by measuring the rate of increase of inorganic phosphate in the reaction mixture.

Preparation of reactants. Crystalline muscle phosphorylase was prepared from rabbit muscle by the method of Green & Cori (1943) but differed from that procedure in two respects. First, the animal was killed by the $MgSO_4$-

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anaesthesia method of Dubois, Albaum & Potter (1943) instead of with Nembutal, as the former ensures that the animal loses consciousness in a relaxed condition. Secondly, instead of using glycerophosphate-cysteine buffers at pH 6·8 of two different strengths, one for the dialysis containing 1 ml. 0·3 M-cysteine-HCl and 39 ml. of 0·03 M-glycerophosphate, and the other for the crystallization of the enzyme containing 0·03 M-cysteine-HCl and glycerophosphate, the procedure adopted by Sumner & Somers (1947), in which the first-mentioned buffer is used for the crystallization, was adopted. In all other respects the directions given by Green & Cori (1943) and not those given by Sumner & Somers were followed.

Glycogen which is used as a primer in the reaction was prepared from rat and rabbit liver by the method of Somogyi (1934). Glucose-1-phosphate was prepared both enzymically from starch with potato phosphorylase according to the method of Hanes (1940), and also by chemical synthesis from glucose by the method of Cori, Colowick & Cori (1937). The Ba salt of glucose-1-phosphoric acid was converted to the dipotassium salt according to the method of Wolfrom & Pletcher (1941).

The reaction mixture. Two tubes were prepared, the one containing the enzyme mixture and the other the substrate mixture. The enzyme mixture (1 ml.) contained sufficient enzyme in a suspension of 0·03 M-cysteine hydrochloride-glycerophosphate buffer at pH 6·7 ± 0·05 to obtain a suitable rate of polysaccharide synthesis from glucose-1-phosphate. The buffer was prepared freshly each day owing to the conversion of the cysteine to cystine in air. Before the addition of the enzyme suspension to the substrate solution the former was incubated at 30° in a water bath for about 15 min. The substrate mixture (1·0 ml.) was prepared by mixing sufficient glucose-1-phosphate solution (K_2 salt at pH 7·0 ± 0·05) with a 5 % solution of glycogen and distilled water so that the concentration of glucose-1-phosphate in the reaction mixture (2 ml.) was between 0·005 and 0·03 M with a glycogen concentration of 1 % (w/v). If it was desired to test the inhibitory effect of any substance, then a solution of the latter replaced part of the distilled water in the substrate mixture. The mixture was incubated in a water bath at 30° for about 15 min. before use.

Method for following the reaction. Before the addition of the enzyme solution to the substrate mixture, 0·1 ml. samples of each were added to 2 ml. of 5 % trichloroacetic acid. Samples of the enzyme solution (0·9 ml.) were then added to 0·9 ml. substrate mixture and the reaction allowed to proceed at 30°. After 5, 10, 25, 40 and 60 min., 0·2 ml. samples of the reaction mixture were withdrawn and added to 2 ml. of 5 % trichloroacetic acid. At the completion of the experiment a further 0·1 ml. sample was withdrawn and added to 2 ml. of distilled water for a determination of the total hydrolysable phosphate in the mixture. The inorganic phosphate content of the samples from the reaction mixture was determined by the method of Fiske & Subbarow (1925), and from this the percentage of the original glucose-1-phosphate which had been converted to polysaccharide was calculated.

Steps in the synthesis of α-3-methyl D-glucopyranose 1-phosphoric acid

Attempts to prepare this substance by the method which Cori *et al.* (1937) employed for α-glucose-1-phosphoric acid were unsuccessful since it was impossible to obtain the required substance from the products of the hydrolysis and deacetylation of tri-(triacetyl 3-methyl D-glucopyranose) 1-phosphoric acid even though a wide range of hydrolysis times were employed. An alternative method of hydrolysis and deacetylation of the intermediate substance has been described by Reithel (1945) for the preparation of β-D-galactopyranose 1-phosphoric acid, but this was also unsuccessful in the present case.

Tetraacetyl 3-methyl β-D-glucopyranose is best prepared by the method of Helferich & Lang (1932) using sodium acetate as the catalyst, although these authors only reported a yield 25 % of the theoretical. By a change in the proportion of the reactants to: 3-methyl glucose 2 parts, sodium acetate 1 part, acetic anhydride 7 parts, instead of 2:1:10 as used by Helferich & Lang, the yield was increased to 46 % of the theory.

An attempt to prepare 2:4:6-triacetyl 3-methyl D-glucosyl bromide from the tetraacetyl derivative by the method described by Gatterman (1941) was not successful. However, the substance required was successfully prepared by a general method kindly communicated to the author by Dr D. J. Bell of Cambridge University. The application of this method in the present case was as follows.

Tetraacetyl 3-methyl D-glucopyranose (8 g.) was added slowly to 16 g. of HBr in glacial acetic acid, the whole being cooled in ice during the addition. After the addition of each small portion of solid the flask was vigorously shaken to promote solution. After solution was complete the flask was allowed to stand at room temperature for 4 hr. About 25 ml. of benzene were added and the contents shaken until a homogeneous solution was obtained. After transferring the contents of the flask to a separating funnel, an equal quantity of ice-water together with some crushed ice was added. The benzene extracts were shaken with water and the water phase removed. The extraction with water was repeated twice, the combined washings extracted with 0·5 vol. of benzene and the benzene extract added to the main benzene fraction. The benzene fraction was then washed twice with an equal volume of a saturated solution of $KHCO_3$, the washings extracted with benzene, and the benzene extract added to the main bulk. The combined benzene fractions were then dried with powdered $CaCl_2$, and the benzene removed under a reduced pressure. The weight of the practically colourless syrup was 7·5 g., $[\alpha]_D^{20°} + 162·8 \pm 0·1$ in $CHCl_3$. A calculation of the specific rotation based on those of α- and β-pentaacetylglucopyranose, tetraacetyl 3-methyl β-D-glucopyranose and tetraacetyl α-D-glucosyl bromide, using the isorotation rules of Hudson (1924), gave a value of +159·5°, which is in comparatively close agreement with that found experimentally, and confirms the characterization of the substance as 2:4:6-triacetyl 3-methyl α-D-glucosyl bromide. The synthesis of this substance was reported by Levene & Raymond (1930), but details of the synthesis and the specific rotation of the product were not reported.

RESULTS

Experiments with yeast

The graph in Fig. 1 is typical of the results obtained for two experiments in which glucose was fermented by yeast with and without 3-methyl glucose. It will be seen that the presence of 3-methyl glucose did not significantly affect the rate of fermentation of glucose by yeast.

Possible inhibition of polysaccharide synthesis from glucose-1-phosphate with crystalline muscle phosphorylase by 3-methyl glucose

Graphs showing the rate of conversion of glucose-1-phosphate to polysaccharide in the presence and absence of glucose and 3-methyl glucose at two different substrate concentrations are shown in Fig. 2 (*a*) and (*b*). The concentration of sugar in the

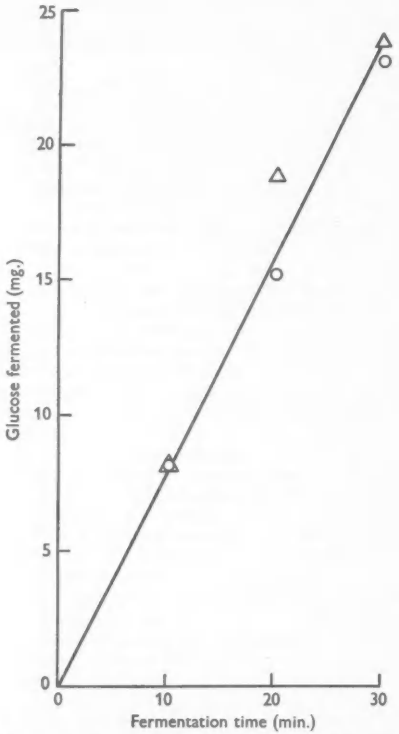

Fig. 1. Effect of 3-methyl glucose on the rate of fermentation of glucose by yeast. The reaction mixtures consisted of 2 ml. yeast suspension, 2 ml. glucose solution (100 mg.) and either 2 ml. water, △, or 2 ml. 3-methyl glucose solution (100 mg.) ○.

reaction mixture in both cases was 0·05 M. It will be seen that the addition of 3-methyl glucose at this concentration to the reaction mixture did not significantly affect the rate of conversion of glucose-1-phosphate to polysaccharide, whereas a similar concentration of glucose very significantly reduced the rate of conversion. Similar results were obtained with a concentration of glucose-1-phosphate of 0·006 M, and with different phosphorylase pre-

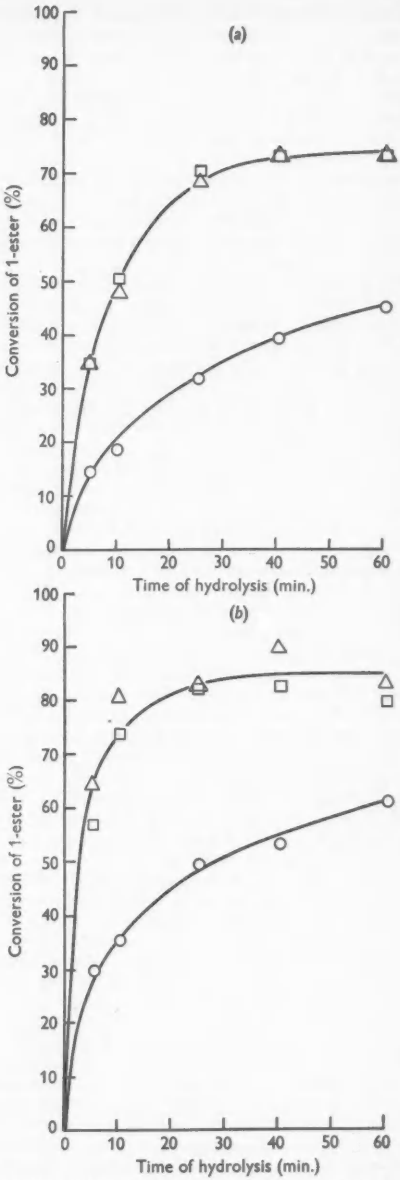

Fig. 2 (*a*) and (*b*). Effect of 3-methyl glucose and glucose on the rate of polysaccharide synthesis from glucose-1-phosphoric acid (K_2 salt) with a crystalline muscle phosphorylase preparation. △, control without added sugar; ○, with 0·05 M-glucose; □, with 0·05 M-3-methyl glucose. Substrate concentration 0·0126 M in (*a*) and 0·006 M in (*b*).

parations. Similar results were also obtained with glucose-1-phosphate prepared both chemically and enzymically.

DISCUSSION

Although 3-methyl glucose is not itself metabolized by yeast the experiments on the rate of fermentation of glucose suggest that the sugar does not even act as a competitive inhibitor in any of the enzyme systems involved in the fermentation of glucose. Colowick (1938) has shown that mannose-1-phosphate and galactose-1-phosphate, and Meagher & Hassid (1946) have shown that maltose-1-phosphate are not substrates for phosphorylase and none of the free sugars of these phosphoric esters inhibits polysaccharide synthesis from glucose-1-phosphate with muscle phosphorylase (Campbell & Creasey, 1949). Indeed, apart from glucose and certain glucosides no other sugar is known to inhibit phosphorylase competitively. It is probable, therefore, that only sugars which inhibit phosphorylase are capable of conversion to polysaccharide with phosphorylase through the intermediary of their 1-phosphate esters. Thus, although it was not possible to determine directly whether 3-methyl glucose-1-phosphate is a substrate for muscle phosphorylase it is unlikely that it is, since the free sugar does not inhibit the synthesis of a polysaccharide from glucose-1-phosphate with phosphorylase. The significance of the failure of 3-methyl glucose to inhibit phosphorylase is considered elsewhere (Campbell, Creasey & Parr, 1952).

SUMMARY

1. 3-Methyl glucose did not significantly affect the rate of fermentation of glucose by yeast.

2. 3-Methyl glucose did not reduce the rate of polysaccharide synthesis from glucose-1-phosphate with a crystalline muscle phosphorylase preparation.

3. It was not possible to prepare 3-methyl glucose-1-phosphoric acid by the methods generally employed for the synthesis of glucose-1-phosphoric acid. However, an improved method for the synthesis of tetraacetyl 3-methyl β-D-glucopyranose is described together with a method for the synthesis of 2:4:6-triacetyl 3-methyl α-D-glucosyl bromide.

It is a pleasure for the author to acknowledge his indebtedness to Prof. F. G. Young, F.R.S., for helpful criticism and advice. The assistance at various stages of the work of Miss Edna Large and Dr N. H. Creasey is also acknowledged with pleasure.

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The Specificity of Muscle Phosphorylase

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In 1940, Cori & Cori showed that glucose inhibited the activity of a purified cat-liver preparation possessing phosphorylase activity, whereas mannose, galactose, maltose, fructose and glucose-6-phosphate had no significant inhibitory effect. Further, from the effect of freshly prepared glucose solutions on the same preparation, they suggested that while α-glucose competitively inhibited the enzyme, β-glucose had little or no inhibitory effect. It seemed probable, therefore, that there was an α, β specificity of the enzyme for the sugar.

In 1943, Green & Cori obtained a crystalline phosphorylase preparation from rabbit muscle. Subsequently, Cori, Cori & Green (1943) showed that the synthesis of polysaccharide from α-glucose-1-phosphate with this enzyme was also competitively inhibited by glucose. In order to determine whether the crystalline enzyme also possessed an α, β specificity the effect of various glucosides, particularly α- and β-methylglucoside, has now been investigated. Moreover, if it is true that a competitive inhibitor of an enzyme combines with the same part of the enzyme surface as its natural substrate, it seemed possible that by determining the inhibitory effect of various sugars and related substances the stereoisomerism and nature of the groups involved in the formation of the enzyme-substrate complex might be elucidated.

METHODS AND MATERIALS

The preparation of the crystalline muscle phosphorylase followed closely the method described by Green & Cori (1943) with the modifications adopted by Campbell (1952). The latter paper also contains a full description of the methods adopted in the present experiments for following the course of the synthesis of polysaccharide from α-glucose-1-phosphate. The concentration of the test substance in the reaction mixture was usually 0·05 M. The inhibitory effect of the test substance was compared with that of a solution of glucose of similar concentration under identical conditions.

The α-glucose-1-phosphate was prepared enzymically from starch with potato phosphorylase according to the method of Hanes (1939).

In the case of the commoner substances whose inhibitory properties were tested laboratory reagent samples were employed. Where special methods were used the following details are given.

1:2-iso*Propylidene*-D-*glucose and* 1:2-5:6-*di*iso*propylidene*-D-*glucose.* These substances were prepared according to the method of Bell (1935).

Gluconic acid. Calcium gluconate (1·12 g.) was dissolved in 10 ml. of water, and 0·4 g. of Na_2SO_4 (anhydrous) was added. The precipitated $CaSO_4$ was filtered off and the pH of the clear solution adjusted to 7·0.

Ascorbic acid. The ascorbic acid solution was brought to pH 6·7 by the addition of N-NaOH before use.

β-*Methyl*-D-*glucoside.* This substance (4 g.) was prepared from 20 g. of acetobromoglucose by the method of Koenigs & Knorr (1901). The product was crystallized from ethanol and an aqueous solution had $[\alpha]_D^{17^\circ} + 32{\cdot}23^\circ \pm 0{\cdot}01$.

α-*Phenylglucoside.* Tetraacetyl α-phenylglucoside was prepared from α-pentaacetyl glucose by the method of Montgomery, Richtmyer & Hudson (1942), and saponified by the technique of Zemplén & Pacsu (1929). The product was crystallized from a mixture of equal proportions of ethanol and ether; an aqueous solution had $[\alpha]_D^{20^\circ} + 172^\circ$.

β-*Glucose*-1-*phosphoric acid* (*Ba salt*). This substance was prepared from acetobromoglucose in 19% yield by the method of Reithel (1945). An aqueous solution of the purified Ba salt had $[\alpha]_D^{20^\circ} + 9{\cdot}4^\circ$.

L-*Talose and* D-*allose.* Samples of these substances were very kindly supplied by Prof. T. Reichstein of Basle University.

RESULTS

The following substances were found to have no significant inhibitory effect on the synthesis of a polysaccharide by the crystalline muscle phosphorylase preparation at a concentration of 0·05 M: D-xylose, D-mannose, D-galactose, D-fructose, L-talose, D-allose, maltose, sucrose, *iso*propylidene-D-glucose, gluconic acid, sorbitol, inositol and ascorbic acid. In addition, di*iso*propylidene-D-glucose failed to show any inhibitory effect at a concentration of 0·02 M.

At a concentration of 0·05 M-α-methylglucoside significantly inhibited the synthesis of a polysaccharide from α-glucose-1-phosphate with the crystalline muscle phosphorylase preparation as compared with a control reaction mixture. The various samples of α-methylglucoside used were found to contain a little free reducing sugar as determined by the Hagedorn & Jensen (1923*a*, *b*)

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estimation. On the assumption that the reducing sugar was glucose a similar amount of glucose was added to the control reaction mixture so that the reducing powers of the control and α-methylglucoside reaction mixtures would be the same.

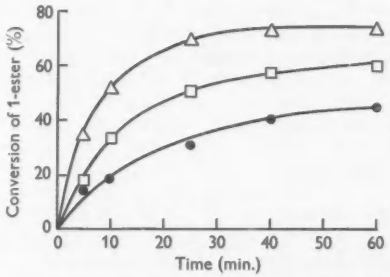

Fig. 1. Inhibition of polysaccharide synthesis by α-methylglucoside and glucose. Crystalline phosphorylase preparation incubated with: α-glucose-1-phosphate, 0·016 M; cysteine-glycerophosphate buffer, 0·015 M, pH 6·8; glycogen 1%. △, +glucose 0·009 M (to compensate for glucose in α-methylglucoside solution); □, +α-methylglucoside 0·05 M; ●, +glucose 0·05 M. At 30°.

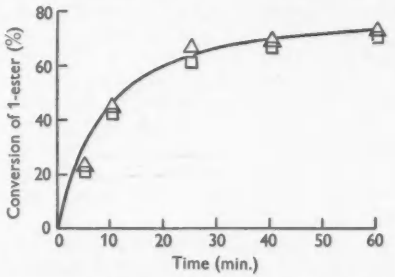

Fig. 2. Failure of β-methylglucoside to inhibit polysaccharide synthesis. Crystalline phosphorylase preparation incubated with: α-glucose-1-phosphate, 0·016 M; cysteine-glycerophosphate buffer, 0·015 M, pH 6·8; glycogen 1%. △, in absence of added sugar; □, in presence of β-methylglucoside 0·05 M. At 30°.

Under similar conditions a 0·05 M solution of β-methylglucoside showed no significant inhibitory effect. These results are shown graphically in Figs. 1 and 2. It will be observed in Fig. 1 that the inhibitory effect of α-methylglucoside is considerably less than that of glucose at a similar concentration. This inhibitory effect was not due to the partial breakdown of α-methylglucoside to glucose, since the reducing power of the reaction mixture did not increase during the course of the reaction. A similar inhibitory effect might also have been caused by a change in the pH during the course of the reaction. However, no significant change in the pH, as

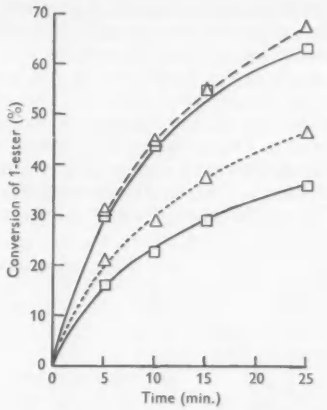

Fig. 3. Effect of concentration of α-methylglucoside on inhibition of polysaccharide synthesis. Crystalline phosphorylase preparation incubated with: α-glucose-1-phosphate, 0·016 M; cysteine-glycerophosphate buffer, 0·015 M, pH 6·8. △, α-methylglucoside 0·1 M; □, α-methylglucoside 0·2 M. The upper curve of each pair is a control containing glucose equivalent in amount to the reducing substance in the α-methylglucoside solution. At 30°.

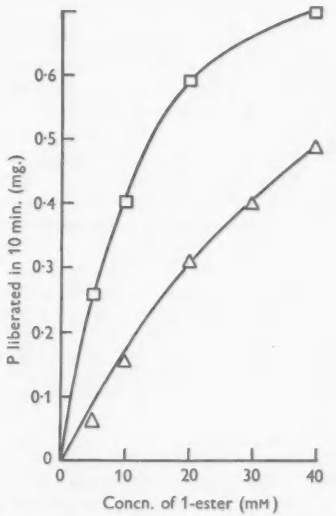

Fig. 4. Effect of substrate concentration on inhibition of polysaccharide synthesis by α-methylglucoside. Crystalline phosphorylase preparation incubated with: α-glucose-1-phosphate, varying concentrations; cysteine-glycerophosphate buffer, 0·015 M, pH 6·8; glycogen 1%. □, in absence of α-methylglucoside; △, in presence of α-methylglucoside, 0·2 M. At 30°.

measured with a glass electrode, took place. It was concluded, therefore, that α-methylglucoside had a direct inhibitory action on the enzyme.

The results shown graphically in Fig. 3 indicate that the inhibitory effect of α-methylglucoside is proportional to its concentration in the reaction mixture. Fig. 4 shows that an increase in the substrate concentration at a given concentration of α-methylglucoside leads to a decrease in the inhibition. This is the type of result which would be

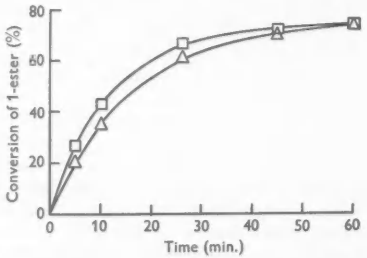

Fig. 5. Effect of α-phenylglucoside on polysaccharide synthesis. Crystalline phosphorylase preparation incubated with: α-glucose-1-phosphate, 0·014 M; cysteine-glycerophosphate buffer, 0·015 M, pH 6·8; glycogen 1 %. □, +glucose 0·25 mM (to compensate for glucose in α-phenylglucoside); △, +α-phenylglucoside 0·05 M. At 30°.

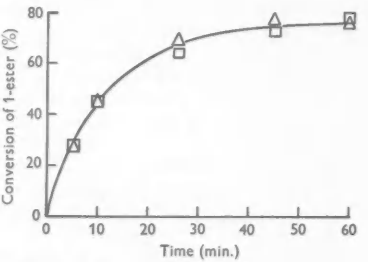

Fig. 6. Effect of β-glucose-1-phosphate on polysaccharide synthesis. Crystalline phosphorylase preparation incubated with: α-glucose-1-phosphate, 0·014 M; cysteine-glycerophosphate buffer, 0·015 M, pH 6·8; glycogen 1 %. △, +glucose 2·0 mM (to compensate for glucose in β-glucose-1-phosphate); □, +β-glucose-1-phosphate 0·03 M. At 30°.

expected if α-methylglucoside were acting as a competitive inhibitor by competing with α-glucose-1-phosphate for the active groups of the enzyme. The maximum velocity of the reaction was determined for different concentrations of α-methylglucoside by the graphical method of Lineweaver & Burk (1934). Although the slope of the line obtained by plotting $1/V$ against $1/[S]$, where V = maximum velocity and S = the substrate concentration, did appear to depend on the inhibitor concentration, it was not possible to show that the maximum velocity remained constant, which should have been the case if the inhibition had been competitive.

That α-phenylglucoside at a concentration of 0·05 M was also slightly inhibitory is shown in Fig. 5.

In order to check whether polysaccharide synthesis by phosphorylase from either α-methylglucoside or α-phenylglucoside was possible, these glucosides were incubated with the enzyme with the usual reaction mixture except for the absence of α-glucose-1-phosphate. Qualitative tests for the presence of amylose, by the addition of iodine solution, were carried out after 1 and 24 hr. incubation. In neither case was there any evidence of the synthesis of a blue-staining polysaccharide with iodine in the reaction mixture, so that it was concluded that neither α-methyl- nor α-phenylglucoside are suitable substrates for phosphorylase.

The effect of β-glucose-1-phosphate is shown in Fig. 6. At a concentration of 0·03 M no significant inhibitory effect was observed.

DISCUSSION

The fact that the α isomer of methylglucoside inhibits phosphorylase, whereas the β isomer does not, confirms the presence of an α, β specificity for phosphorylase, a fact that was originally suggested by Cori & Cori (1940) as a result of their experiments with a freshly prepared solution of glucose. This α, β specificity is further confirmed by the results obtained with α-phenylglucoside and β-glucose-1-phosphoric acid. In the latter case it seems that not only is the β isomer an unsuitable substrate for phosphorylase, but that it also fails to act as a competitive inhibitor.

The inability to demonstrate rigorously that α-methylglucoside competitively inhibits phosphorylase is probably due to the complex nature of the reaction mixture. Cori *et al.* (1943) have shown that while cysteine increases the activity of phosphorylase, sodium glycerophosphate acts as an inhibitor and since both these substances were present in the reaction mixture used in the present experiments, the effect of any other inhibitor must be complicated. It would seem desirable in any further work to arrange the conditions so that there is only one inhibitor present in the reaction mixture, e.g. by the use of a solution of KCl instead of sodium glycerophosphate (Green & Cori, 1943).

It is believed that a competitive inhibitor combines with the same active group of an enzyme as does its natural substrate, but with the formation of an inactive, instead of an active, enzyme complex. As far as is known, the only substance that will form an active enzyme complex with phosphorylase, that is one leading to polysaccharide synthesis, is α-D-glucose-1-phosphate. However, other gluco-

sides are able to unite with the active centres of phosphorylase, as is shown in these experiments by the fact that polysaccharide synthesis from α-D-glucose-1-phosphate is inhibited when the aglycone consists of hydroxyl (α-D-glucose), methyl or phenyl radicals, affinity for the enzyme decreasing in the order given. It is assumed that the enzyme complex formed with these glucosides is inactive since it was not possible to demonstrate the synthesis of a blue-staining polysaccharide with iodine when the enzyme was incubated with the glucosides in the absence of α-glucose-1-phosphate. If the aglycone be glucose (maltose) or fructose (sucrose) no inhibition occurs. Possibly the size of the aglycone is important in this gradual loss of affinity.

The effect of ascorbic acid was tested, for Gill & Lehmann showed in 1939 that this substance inhibited the formation of Robison ester from glycogen with a crude rabbit-muscle extract showing phosphorylase activity. However, no evidence was obtained from the present experiments that ascorbic acid had any significant effect on the rate of polysaccharide synthesis from α-glucose-1-phosphate with a crystalline muscle phosphorylase preparation.

Apart from the glucosides, of the substances tested only glucose inhibited phosphorylase. Since sorbitol and inositol were without effect a pyranose structure appears to be necessary for the substance to be inhibitory. The failure of D-xylose indicates the necessity for a primary alcohol group on $C_{(5)}$, and that of D-mannose, D-galactose, D-allose and D-talose that the configuration of the groups attached to $C_{(2)}$, $C_{(3)}$ and $C_{(4)}$ must be as in glucose (cf. Gottschalk, 1947, 1950). The importance of the configuration of the groups attached to $C_{(5)}$ in glucose could be tested if β-L-idose were available, for this sugar has exactly the same configuration as α-D-glucose except on $C_{(5)}$ where the position of the attached group is reversed. The effect of substituting a methoxyl for a hydroxyl group in glucose has already been demonstrated by the lack of inhibitory effect of 3-methyl glucose (Campbell, 1952).

The failure of D-fructose to inhibit phosphorylase can be interpreted in a number of ways. First, any furanose form of this sugar which may be present in solution would understandably not act as an inhibitor as its ring C atoms would be planar and not somewhat kinked as in a pyranose structure. Secondly, on the basis that $C_{(1)}$ of D-glucopyranose should be compared with $C_{(2)}$ of D-fructopyranose the configuration of the groups attached to the C atom is different in the two molecules. Thirdly, there is a primary alcohol group on $C_{(5)}$ of glucose but not on $C_{(6)}$ of fructose. Finally, Pigman & Goepp (1948) suggest that it is the β isomer of D-fructose which predominates.

It has been shown that the substitution of the hydroxyl groups on $C_{(2)}$, $C_{(3)}$, or $C_{(4)}$ by methoxyl or tosyloxy groups renders a β-glucoside resistant to the action of many β-glucosidases (Helferich & Grünler, 1937; Pigman & Richtmyer, 1942). However, it is possible to make substitutions by replacement of OH by H, F, Br or Cl at $C_{(6)}$ of β-glucosides without rendering the glucoside resistant to hydrolysis by β-glucosidases (Helferich, Grünler & Gnüchtel, 1937). It might be thought that the same consideration would apply in the case of substitutions on $C_{(6)}$ of glucose and its ability to combine with phosphorylase. Experiments designed to check this possibility would be of great interest, although Cori & Cori (1940) have shown that glucose-6-phosphate did not inhibit a purified cat liver phosphorylase preparation.

It seems, therefore, that the only substances which are able to combine with the active centres of muscle phosphorylase are α-D-glucopyranose and certain glucosides, with the possible exceptions already mentioned. In no circumstances can the aglycone of an inhibitory glucoside be another sugar molecule, except of course in the case of glycogen and similar polysaccharides which are degraded by phosphorylase.

SUMMARY

1. The following substances have been shown to have no significant inhibitory effect on the synthesis of a polysaccharide from α-glucose-1-phosphoric acid with a crystalline muscle phosphorylase preparation under the experimental conditions used: D-xylose, D-mannose, D-galactose, D-fructose, L-talose, D-allose, maltose, sucrose, *iso*propylidene-D-glucose, di*iso*propylidene-D-glucose, gluconic acid, sorbitol, inositol, ascorbic acid, β-glucose-1-phosphoric acid and β-methylglucoside.

2. Under identical experimental conditions the following substances significantly inhibited the reaction: D-glucose, α-methylglucoside and α-phenylglucoside.

3. The significance of these results in the elucidation of the specificity of phosphorylase is discussed.

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Studies on the Biochemistry of Zinc

3. FURTHER INVESTIGATIONS ON THE INFLUENCE OF ZINC ON METABOLISM

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Changes in the liver and bones and in the metabolism of nitrogen, phosphorus and sulphur in rats maintained on a stock diet supplemented with zinc have already been reported (Sadasivan, 1951*a*, *b*). It was also observed that on a high-fat, low-protein diet, zinc supplements caused a lowering in fat content of rat livers. In this paper, results are presented of investigations on the metabolism of nitrogen, phosphorus and sulphur and the urinary excretion of uric acid and total creatinine (creatine and creatinine) in rats fed on a high-fat, low-protein diet supplemented with 0·5 and 1·0% of zinc oxide. Results of experiments on the influence of the element on the assimilation of fat from the intestines and on the activity of the phosphatases and lipase in the system are also included.

EXPERIMENTAL

Metabolism experiments. The details of the metabolism experiments have already been described in the previous paper in this series. In the present experiments, the high-fat, low-protein diet described in Part 1 was fed to rats. The daily food intake was recorded and calculated for each 3-day period. The collection of urine, faeces, etc., and their analyses were carried out in the same manner as described in Part 2 (Sadasivan, 1951*b*). The first three groups of young rats weighed about 40–50 g. each and were about a month old, while groups 4 and 5 were older ones weighing between 90 and 100 g. and 2–2·5 months old. Younger and older rats were compared because preliminary observations suggested that younger rats are probably more susceptible to the effects of zinc than older ones.

For the experiment on fat metabolism, each group consisted of six rats equally distributed between the sexes and litters. Both the groups were fed the high-fat diet, the second group receiving, in addition, ZnO at 0·5% level. The faeces were collected for each 3-day period over the experimental period of 15 days and the fat content of the dried faeces was determined by extraction with ether in a Soxhlet extractor. For the determination of fat in the liver, the animals were killed at the end of the experiment. The liver was removed, freed of blood by pressing lightly against blotting paper and ground with a small amount of acetone in a mortar. Fat was then extracted in a Soxhlet extractor with acetone.

Determination of phosphatase activity. Two groups of six rats, each weighing between 60 and 80 g., were fed the stock diet described in Part 1. The second group received, in addition, ZnO at 1·0% level of diet. At the end of 15 days, the animals were killed and liver, small intestines, kidneys and femurs were removed for the determination of phosphatase activity. The tissues were freed of blood by washing once with distilled water and femurs cleaned of adhering flesh. Portions of the tissues and the whole of the femurs were prepared for determination of activity according to the method of Bodansky (1937) and of Martland & Robison (1929). Activity was measured in sample portions of the material suspended in 0·033 M-veronal-acetate buffer adjusted to a pH of 9·1. Sodium β-glycerophosphate at concentrations of 1·0% served as substrate and the mixture

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was incubated at 37° for 1 hr. at the end of which inorganic P was determined by the method of Fiske & Subbarow (1925) with due correction for the presence of phosphate in the crude enzyme preparations by running parallel controls in all cases.

Determination of lipase activity. An active preparation of pancreatic lipase of rats was prepared by treating fresh, minced pancreas from healthy rats with an excess of pure acetone, grinding in a mortar and filtering after contact with acetone for about half an hour. The process was repeated twice and the residue dried *in vacuo*. This powder was extracted with glycerol for 24 hr., filtered through muslin and the extract used as the source of the enzyme. To test the activity, 2 ml. portions of a 5% extract were added to 5 ml. 0·033 M-veronal-acetate buffer adjusted to the pH required and 4 ml. of pure olive oil added as substrate. After the additions had been made, the final volume was made to 15 ml. with water and the mixture incubated at 37° for 3 hr. after which 25 ml. of neutralized ethanol were added and the acid liberated by the enzyme determined by titration against 0·1 N-NaOH using phenolphthalein as the indicator. $ZnSO_4$, NaCN, $CaCl_2$ etc., used in the experiments, were of Merck's purest grade.

RESULTS AND DISCUSSION

Metabolism on a high-fat diet. Table 1 summarizes the results of metabolism of nitrogen, phosphorus, etc., obtained for the duration of the experiment when the animals were fed a high-fat, low-protein diet supplemented with zinc. It may be concluded from these results that on a high-fat, low-protein diet, the changes in metabolism of the various constituents studied are similar to those on a stock diet reported in the previous paper. The adverse effect of zinc supplements on the assimilation of phosphate from the intestines does not appear to be due to the precipitation of phosphate in the insoluble form as in beryllium rickets (Kay & Skill, 1934). The figures in Table 2 show that the quantities of zinc ingested in the animals in the different groups were much less than required to precipitate the phosphate completely. Thus, though a fairly good proportion of the phosphates in the synthetic diet

Table 1. *Influence of dietary zinc on the excretion and retention of* N, P *and* S *and on the excretion of uric acid and total creatinine by rats fed on a high-fat, low-protein diet*

(Summary of results for the whole period of experiment. All values expressed as mean ± S.E.)

	Young rats			Older rats	
	Control	Zinc fed at 0·5% level as ZnO	Zinc fed at 1·0% level as ZnO	Control	Zinc fed at 0·5% level as ZnO
Food intake (g.)	57±8·3	37±2·0	30±1·7	69±0·7	67±1·1
Change in body weight (g.)	+5±4·4	−5±4·7	−11±1·3	+0·5±2·1	+0·5±1·9
Metabolism of nitrogen (mg.):					
Urinary excretion	62±4·5	71±14·1	78±11	142±31	100±11
Faecal excretion	65±8·4	93±4·3	97±6	103±8	151±5
Retention	578±91	311±34	195±18	600±64·5	584±58
Metabolism of phosphorus (mg.):					
Urinary excretion	30±3·6	20±3·9	16±3·7	70±12·2	36±3·5
Faecal excretion	21±1·5	49±7·8	71±9·4	32±3·0	125±11
Retention	232±37·9	114±7·1	61±6·2	241±28	155±16·1
Metabolism of sulphur (mg.):					
Urinary excretion	10·2±0·17	9·6±0·2	8·8±0·14	8·8±1·43	8·12±1·1
Faecal excretion	15·4±0·55	18·2±0·55	19·4±0·65	18·5±0·6	26·3±1·8
Retention	−1·3±3·1	−12·0±0·8	−15·9±1·0	2·8±2·6	−4·9±0·6
Uric acid in urine (mg.)	1·61±0·23	1·57±0·54	1·0±0·79	4·58±1·7	−4·9±0·6
Total creatinine in urine (mg.)	11·85±7·1	8·73±3·8	6·87±0·9	12·6±2·9	10·83±0·9

Table 2. *Quantities of zinc and phosphorus in the diet of rats supplemented with zinc*

(High-fat, low-protein diet in all groups.)

Group no.	Supplement to diet	Intake of food during 15 days (g.)	Intake of ZnO in diet (mg.)	Phosphorus in diet (mg.)	Amount of P precipitable by Zn in diet (mg.)
2	0·5% ZnO	36·65	190·6	183·25	47·65
3	1·0% ZnO	29·70	305·9	148·50	76·48
5	0·5% ZnO (older rats)	67·00	348·4	335·00	87·10

remained in an assimilable form, the quantity actually assimilated appeared small.

Zinc and phosphatase activity. The decrease in the assimilation of phosphorus from the intestines and the poor development of the bones consequent on the intake of zinc in the concentrations studied suggest that zinc affects adversely some process of phosphorus metabolism. To check this point, the phosphatase activity of the tissues of zinc-fed animals was compared with that of normal ones. The results (Table 3) show that the intake of zinc at the level investigated brings about a significant decrease in phosphatase activity of the intestine while increasing it in the liver and kidneys. The difference in the activity of the bones does not appear to be significant (cf. Hove, Elvehjem & Hart, 1940).

Table 3. *Phosphatase activity of tissues of rats fed on a stock diet supplemented with zinc*

(For method see text. Results as mean ± S.E.)

	P liberated (mg.)	
	Control	Zinc-fed
Intestines	0·615 ± 0·04	0·499 ± 0·01
Liver	0·069 ± 0·005	0·116 ± 0·005
Bones (femur)	0·103 ± 0·007	0·133 ± 0·019
Kidneys	0·253 ± 0·01	0·477 ± 0·02

Zinc and fat metabolism. The importance of phosphorylation in the absorption of triglyceride fat from the intestines has been investigated by several workers, and the literature on the subject has been reviewed by Frazer (1946). If assimilation of fat is closely related to and dependent on its phosphorylation in the intestines, the impairment of phosphate assimilation from the organ should result in an increase in the faecal fat with or without a simultaneous decrease in the fat intake. In our experiments no appreciable difference in the faecal excretion of fat could be observed, it being 0·84 g. ± 0·05 and 0·71 g. ± 0·05 per rat in the control and zinc-fed groups respectively for the experimental period of 15 days. The corresponding figures for fat intake during the period were 29·3 g. ± 1·08 and 21·3 g. ± 1·36 and for fat content of liver, 1·69 g. ± 0·1 and 0·68 g. ± 0·09.

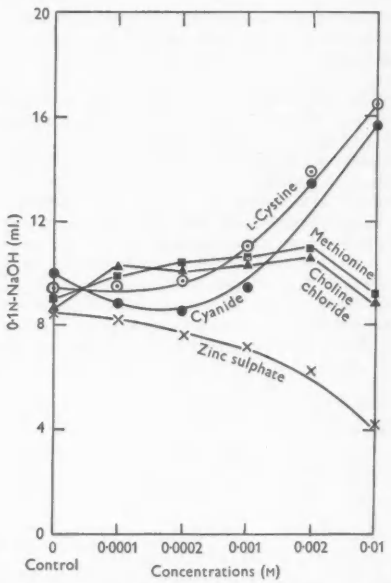

Fig. 1. Influence of zinc, cystine, NaCN, etc., on the lipolytic activity of pancreatic lipase of rats at pH 8·2 and in the presence of Ca^{++} ions.

Zinc and lipolytic activity of pancreatic lipase. The influence of zinc on the fat content of liver was further investigated by testing its effect on the lipolytic activity of pancreatic lipase, and the results of experiments in which zinc sulphate, sodium cyanide and L-cystine were added at a concentration of 0·01 M to pancreatic lipase with and without prior addition of calcium chloride and at two different pH values (6·8 and 8·2) are presented in Table 4. In another experiment, the effect of different concentrations of these substances and

Table 4. *Influence of zinc and cystine on the lipolytic activity of pancreatic lipase of rats*

(For method see text.)

	(ml. 0·1 N-NaOH)	
	pH 8·2	pH 6·8
Control	11·4	7·8
Control + 0·01 M-NaCN	15·0	14·4
Control + 0·01 M-$ZnSO_4$	12·6	4·8
Control + 0·01 M-L-cystine	15·9	7·5
Control + 20 mg. $CaCl_2$	20·7	5·4
Control + 20 mg. $CaCl_2$ + 0·01 M-NaCN	30·9	14·1
Control + 20 mg. $CaCl_2$ + 0·01 M-$ZnSO_4$	13·2	8·4
Control + 20 mg. $CaCl_2$ + 0·01 M-L-cystine	29·4	6·0

also of the two well known lipotropic factors, choline and methionine, on the activity of the enzyme at a pH of 8·2 in the presence of Ca^{++} ions was tested. The results, presented in Fig. 1, show clearly that both cystine and cyanide increase the lipolytic activity of the enzyme under the conditions specified. This effect of cystine may be related to its known influence on the accumulation of fat in the liver of rats fed on a diet low in lipotropic factors (Beeston & Channon, 1936; Tucker & Eckstein, 1937; Mulford & Griffith, 1942). The significance of results obtained when zinc and cyanide were added is not at present clear and merits further investigation.

SUMMARY

1. The changes in the metabolism of nitrogen, phosphorus and sulphur and in urinary excretion of uric acid and total creatinine (creatine and creatinine) in rats fed on a high-fat diet supplemented with zinc have been shown to be similar to those observed for a stock diet. It has also been shown that the decreased assimilation of phosphate from the intestines is not due to its being precipitated as insoluble zinc phosphate.

2. Zinc supplements at the levels investigated affected phosphatase activity of the tissues. While the activity of intestinal alkaline phosphatase was lowered, there was an increase in activity in the liver and kidneys in the supplemented groups.

3. Though zinc supplements lowered the fat content of the liver and the assimilation of phosphate from the intestines, there appeared to be no change in the excretion of fat in the faeces.

4. The influence of zinc and other substances such as choline, methionine, L-cystine and cyanide on the lipolytic activity of pancreatic lipase of the rat was also investigated.

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Some Biochemically Relevant Properties of *N*-Substituted Fructosamines Derived from Amino-Acids and *N*-Arylglucosylamines

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N-Substituted glucosamines (*N*-acetyl-D-glucosamine, *N*-formyl-D-glucosamine, *N*-methyl-L-glucosamine), derived from 2-amino-2-deoxyglucose, are well known as components of polysaccharides and mucoproteins produced by animal cells, fungi and bacteria. Fructosamine (1-amino-1-deoxyfructose) (also called *iso*glucosamine) and *N*-substituted fructosamines have not been found among the hydrolytic products of polysaccharides and mucoproteins. It was, however, recently shown (Gottschalk, 1951) that an enzyme present at the surface of the influenza virus splits off from a homogeneous mucoprotein, isolated from human urine (Pye, to be published), an *N*-substituted fructosamine characterized as a condensation product of a ketose and an amino-acid.

N-Substituted fructosamines (III, p. 459) are prepared chemically from the so-called *N*-glucosides of primary aromatic amines (*N*-arylglucosylamines, I, p. 459) through the Amadori rearrangement. Thus *N*-arylglucosylamines from *p*-toluidine, *p*-phenetidine and 3:4-dimethylaniline are transformed to the corresponding fructosamines when heated for a few hours in ethanolic solution with a weak acid as catalyst (Kuhn & Weygand, 1937; Weygand, 1939, 1940). The isomerization of glucosylamines to fructosamines, i.e. the rearrangement of a D-glucose to a D-fructose derivative, has been observed with *N*-arylglucosylamines only. So far attempts to induce the Amadori rearrangement in *N*-alkylglucosylamines have failed (Mitts & Hixon, 1944). While continuing our investigations into the inter-

action between simple sugars and amino-acids (Gottschalk & Partridge, 1950), with the aim of preparing in quantity *N*-glucosylamino-acids (so-called amino-acid *N*-glucosides) it became evident that by boiling for several hours anhydrous D-glucose and DL-phenylalanine in thoroughly dried methanol the *N*-substituted fructosamine, fructose-phenylalanine was formed. (III, $R=CH(CO_2H).CH_2.C_6H_5$; the systematic name, '*N*-(1'-carboxy-2'-phenylethyl)amino-1-deoxyfructose' is so clumsy that the trivial name 'fructose-phenylalanine' is used in this paper.) The compound was practically free from contamination by *N*-glucosyl-phenylalanine which must be assumed to be the first product of reaction, its carboxyl group providing the acidic catalyst required for the Amadori rearrangement; it is known that glucosylamino-acids are stronger acids than the respective free amino-acids (Micheel & Klemer, 1951). Earlier preparations of *N*-glucosylamino-acids obtained by a similar technique probably also consisted mainly of the isomeric fructosamine without it having been recognized. Fructose-phenylalanine has not been crystallized and it was not possible to free it completely from impurities. The chromatographic and analytical results, however, suggest that the amount of contaminants is small. Moreover, *N*-*p*-tolyl-D-fructosamine, a crystalline *N*-arylfructosamine, was found to have in common with the phenylalanine analogue certain very characteristic reactions. On examination of their behaviour towards mineral and organic acids the *N*-substituted fructosamines tested revealed properties differentiating them clearly from *N*-substituted glucosamines and from the parent D-fructose thus providing a tool for the detection of such structures in natural products.

EXPERIMENTAL

Chromatography

General. Paper partition chromatography was carried out in the usual way with Whatman no. 1 paper. The chromatograms were developed for 16 hr. with the upper phase of a mixture of *n*-butanol, acetic acid and water in the ratio 40:10:50 (by vol.), the lower phase serving as stationary phase (Partridge, 1948). The assays were applied to the paper with a calibrated capillary pipette (0·005 ml.). After development, the paper strips or sheets were freed of the solvent by exposing them to a strong draught in the fume chamber for several hours at room temperature followed by 10 min. heating at 95° in the drying chamber. Owing to variations in the room temperature and to an uncontrolled degree of esterification of the solvent, the R_F values quoted were not constant and refer only to a given set of conditions.

Colour reactions used on paper. Phenylalanine was located with ninhydrin, glucose with aniline hydrogen phthalate (Partridge, 1949), 5-(hydroxymethyl)-2-furaldehyde with a solution consisting of 10 ml. 1% (w/v) resorcinol in ethanol and 90 ml. 2 N-HCl. To locate *p*-toluidine use was made of the observation that *p*-dimethylaminobenzaldehyde (*p*-DAB) in the presence of HCl gives an intense yellow colour with *p*-toluidine. For use on paper a solution was prepared containing 80 mg. *p*-DAB (recrystallized) in a mixture of 3·0 ml. conc. HCl and 3·0 ml. ethanol; for spraying 5·0 ml. solution were mixed with 15·0 ml. purified *n*-butanol. The *N*-substituted fructosamines reacted on paper with both aniline hydrogen phthalate and the appropriate reagent for the amine. The Schiff's bases of 5-hydroxymethylfurfuraldehyde were traced by their colour reactions with resorcinol and with the reagent for the amine.

Semiquantitative analysis. For the semiquantitative determination by chromatography of glucose, phenylalanine, *p*-toluidine, 5-hydroxymethylfurfuraldehyde, *N*-*p*-tolyl-D-fructosamine and fructose-phenylalanine the assays were run and compared with serial dilutions of the appropriate reference substance. These dilutions were adjusted in such a way that the concentration of the critical standard and that of the assay did not differ by more than 10% as judged by the area of the spot and by the colour intensity. When the colour given by the assay was intense, a dilution was made so as to bring the colour intensity into a range where a 10% difference could be appreciated. All chromatograms for quantitative evaluation were run at least twice on successive days. For this procedure an accuracy greater than $\pm 10\%$ is not claimed. Because of the volatility of *p*-toluidine at higher temperatures this compound both as standard and in the assay was run on paper as *p*-toluidine hydrochloride.

Other methods used. Nitrogen determinations were made by the micro-Kjeldahl method using the apparatus of Parnas & Wagner; the period of combustion was 8 hr. after clearing; the catalyst mixture consisted of K_2SO_4, $CuSO_4.5H_2O$ and sodium selenate as described by Chibnall, Rees & Williams (1943). The reducing power was determined by the Hagedorn-Jensen micromethod.

Purification of n-*butanol.* The commercial product was refluxed for 24 hr. over NaOH, then distilled and the distillate after drying over anhydrous Na_2SO_4 fractionated in a column; the fraction boiling at 117–118° was collected.

Drying of methanol. Methanol (200 ml.) was slowly added to a mixture of 10 g. powdered Mg and 0·5 g. I_2; when the reaction ceased, further 800 ml. methanol were added, the mixture refluxed for 6 hr. and the dry methanol separated by distillation.

N-p-*Tolyl-*D-*glucosylamine.* This was prepared according to Weygand (1939); *N*-*p*-tolyl-D-fructosamine according to Kuhn & Dansi (1936) and 5-hydroxymethylfurfuraldehyde according to Haworth & Jones (1944).

Fructose-phenylalanine

Preparation. Dried methanol (140 ml.) was added to 700 mg. DL-phenylalanine and boiled under reflux for 1 hr., when only part of the amino-acid was dissolved. Anhydrous D-glucose (5 g.) was then added and the mixture refluxed for 4–5 hr.; 1 hr. after the addition of glucose nearly all phenylalanine was dissolved indicating the progress of the condensation reaction. After a while the methanolic solution turned yellow and later on light brown; at this stage heating was interrupted. After cooling, the solvent was removed *in vacuo* at 35°. The brown residue was dissolved in 75 ml. water, 5 g. thoroughly washed and freshly compressed baker's yeast added and the suspension stirred mechanically at 26° for 7 hr., when the excess glucose was completely fermented as

ascertained by chromatography. The yeast was centrifuged off, the supernatant filtered and the filtrate concentrated under reduced pressure and finally dried in a desiccator over P_2O_5. The resulting brittle material which could easily be powdered with a glass rod was dissolved in 10·0 ml. methanol, a white precipitate (phenylalanine) removed, the solvent evaporated by a fan at 20° and the residue dried again in the desiccator. This procedure was repeated once more. Yield 1·34 g. Attempts to crystallize the substance failed.

Chemical analysis. A stock solution was prepared by dissolving 1·3 g. of dried material in water to a final volume of 10 ml.; pH was 4·0. After removal of some insoluble dark matter this solution was analysed qualitatively and semi-quantitatively by chromatography and quantitatively for N and reducing power. Chromatography revealed (1) a spot with R_F 0·43 reacting with both ninhydrin and aniline hydrogen phthalate, the ninhydrin spot becoming more marked when the paper was dried at 95° instead of at room temperature, (2) a ninhydrin-reacting spot with R_F 0·59 coinciding with the phenylalanine standard and equivalent to a total content of 33 mg. phenylalanine in the original preparation and (3) a faint spot reacting with aniline hydrogen phthalate (brown colour) and trailing from R_F 0·31 to R_F 0·41. No glucose spot was detectable though even 5 mg. (in total) would have been traced. N content: 45·0 mg. Reducing power (as glucose): 532 mg. (both for total original solution). N and reducing power were determined in an appropriate dilution of the stock solution. The stock solution strongly reduced Benedict's reagent on heating and gave a positive Seliwanow test; in alkaline medium *o*-dinitrobenzene and methylene blue were reduced in the cold.

Since the chromatograms indicated the presence only of two ninhydrin-reacting spots and as ninhydrin is an effective reagent not only for amino-acids but also for primary aliphatic amines and even for some secondary aliphatic amines (Bremner & Kenten, 1951), the total amount of fructose-phenylalanine can be computed from the chromatographic and analytical data. Since 33 mg. free phenylalanine containing 2·8 mg. N were recovered, there are present in total original solution $42·2 \times 327/14 = 986$ mg. fructose-phenylalanine, i.e. 71% of theory. 986 mg. compound contain the equivalent of 543 mg. glucose; the experimental figure of 532 mg. glucose compares well with that to be expected from the N analysis. It may be mentioned that in another preparation free of the trailing spot described under (3)—but containing more free phenylalanine—the reducing power of the compound was 92% referred to its glucose equivalent, whereas in the above preparation it is 98%; apparently the substance responsible for the trailing spot contributes about 5% to the total reducing power. The reducing power of the glucose residue in *N*-acetyl-D-glucosamine was found to be 89% (Hagedorn-Jensen; glucose = 100).

Colour reaction with p-*DAB.* When 1·0 ml. of a solution containing 5 mg. compound was heated with acetylacetone in alkaline medium followed by the addition of *p*-DAB (Elson & Morgan, 1933), a purple colour was produced identical in shade with that given by a glucosamine standard but of only about 10% its intensity, if equimolar amounts are compared. Spectroscopic examination (Hartridge spectrometer) disclosed that the positions of the absorption bands produced with D-glucosamine and with the *N*-substituted fructosamine were practically identical: they were 537 and 539 mμ. respectively.

N-p-*Tolylfructosamine.* A 2% (w/v) solution of *p*-tolylfructosamine (in dilute acetic acid) was submitted to chromatography: a spot with R_F 0·74 reacting with both aniline hydrogen phthalate and *p*-DAB was obtained.

The substance strongly reduced Benedict's reagent in the hot and *o*-dinitrobenzene and methylene blue (in alkali) in the cold, as described (Kuhn & Weygand, 1937; Kuhn & Birkofer, 1938). The Seliwanow test, if carried out as is usually done in 12% final concentration of HCl was negative; when performed in ethanol saturated with HCl gas in the cold, the test was positive. In the Elson-Morgan test the compound gave a red colour immediately on adding *p*-DAB; however, on shaking, the yellow colour formed by the reagent with *p*-toluidine prevailed resulting finally in a brownish colour.

N-p-*Tolyl*-D-*glucosylamine.* A 2% (w/v) solution of the glucoside (in water) was submitted to chromatography; there were observed a spot with R_F 0·18 trailing to R_F 0·27 reacting with aniline hydrogen phthalate (glucose standard R_F 0·18) and a spot with R_F 0·85 reacting with *p*-DAB and coinciding with the spot given by *p*-toluidine. Obviously the *N*-glucoside is completely hydrolysed under these conditions.

N-*p*-Tolyl-D-glucosylamine (0·3% w/v in water) containing glucose and *p*-toluidine residues corresponding to 0·201% glucose and 0·119% *p*-toluidine were found to have a reducing power corresponding to 0·239% of glucose; since 0·2% (w/v) *p*-toluidine (in water) has a reducing power corresponding to 0·072% of glucose, the corrected figure for the glucose present in the glucoside is $0·239 - 0·043 = 0·196$%, i.e. 97·5% of the glucose equivalent of *N*-*p*-tolyl-D-glucosylamine.

The *N*-glucosylamine derivative reduced Benedict's reagent in the hot, but not *o*-dinitrobenzene in the cold; it gave a positive Seliwanow test in the modification mentioned above. In the Elson-Morgan test the compound showed a behaviour similar to that of *N*-*p*-tolylfructosamine, though the intensity of the colour formed was less.

Treatment of N-*substituted fructosamines and of* N-p-*tolylglucosylamine with acid*

Experiment 1. To 3·0 ml. of a solution containing approx. 100 mg. substance were added 0·33 ml. conc. HCl and 0·40 ml. glacial acetic acid, the tubes were immediately sealed and submerged for 2 hr., in a boiling water bath. The solutions, after cooling, were neutralized with a measured quantity of 10N-NaOH, filtered and submitted to semi-quantitative analysis by chromatography. The results are shown in Table 1 and Fig. 1.

As indicated in Fig. 1 the acetic acid-treated sample of *N*-*p*-tolylfructosamine contained a small amount of the Schiff's base of 5-hydroxymethylfurfuraldehyde. In the case of fructose-phenylalanine the corresponding Schiff's base (R_F 0·62) was found occasionally as impurity in the preparation of the original compound but not after acid treatment, probably due to hydrolysis of the Schiff's base under these conditions.

A better separation of 5-hydroxymethylfurfuraldehyde and *p*-toluidine than shown in Fig. 1, *D*5 and *D*6, was obtained when the assay was subjected to electrophoresis on paper using an apparatus similar to that described by Cremer & Tiselius (1950). The analysis was carried out in 0·1N-acetic acid at 20° applying a potential of 180 V. for 4 hr.

Table 1. *Action of* HCl *and acetic acid on* N*-substituted fructosamines and an isomeric* N*-glucosylamine derivative*

Substrate and treatment	Substrate initial (mg.)	Substrate left at end of experiment	D-Glucose liberated (mg.)	5-Hydroxy-methyl-furfural formed (mg.)	Amine released (mg.)	Insoluble humin formed
Fructose-phenylalanine:						
N-HCl, 100°, 2 hr.	98·6	42·9	0	6·8	25·9	+
2N-Acetic acid, 100°, 2 hr.	98·6	9·9	2·5	20·4	25·9	Trace
N-p-Tolylfructosamine:						
N-HCl, 100°, 2 hr.	100·0	50·0	0	6·6	20·0	+
2N-Acetic acid, 100°, 2 hr.	100·0	0	0	17·0	20·4	+ + +
N-p-Tolylglucosylamine:						
N-HCl, 100°, 2 hr.	100·0	0	59·9	<1·5	40·2	Trace
2N-Acetic acid, 100°, 2 hr.	100·0	0	16·7	13·3	25·4	+ + +

Experiment 2. 70·0 mg. D-Fructose, dissolved in 3·40 ml. 2N-acetic acid, were heated in a sealed tube for 2 hr. at 100°. This treatment did not result in the formation of enough 5-hydroxymethylfurfuraldehyde to be detectable by chromatographic analysis.

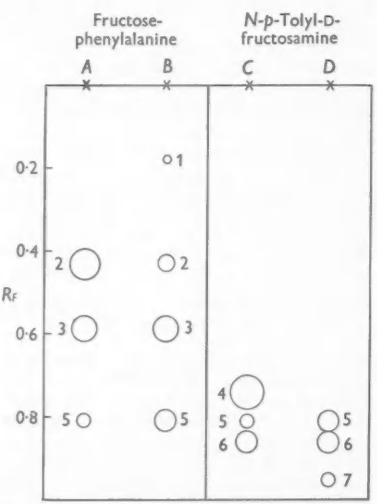


Fig. 1. Chromatograms of acid-treated *N*-substituted fructosamines. *A* and *C*: compounds treatedwith N-HCl. *B* and *D*: compounds treated with 2N-acetic acid. (1) glucose; (2) fructose-phenylalanine; (3) phenylalanine; (4) *N-p*-tolylfructosamine; (5) 5-hydroxymethylfurfuraldehyde; (6) *p*-toluidine; (7) Schiff's base of (5) with (6).

Experiment 3. In two experiments were added to 3·0 ml. 0·6% (w/v) solution of 5-hydroxymethylfurfuraldehyde 0·33 ml. conc. HCl and 0·40 ml. glacial acetic acid respectively and the mixtures heated in sealed tubes for 2 hr. at 100°. Recoveries of the compound by chromatography were 25 and 100% respectively.

Alkaline treatment of N*-substituted fructosamines*

Experiment 4. A solution containing approx. 300 mg./100 ml. (w/v) fructose-phenylalanine (in water) was tested for its reducing power before and after treatment with 0·1N-Na_2CO_3 (final concn.) at 100° for 20 min.; the same was done with a solution containing 140 mg./100 ml. (w/v) D-glucose (in water). With the former compound the reducing power decreased by 72·9%, with glucose by 77·5%.

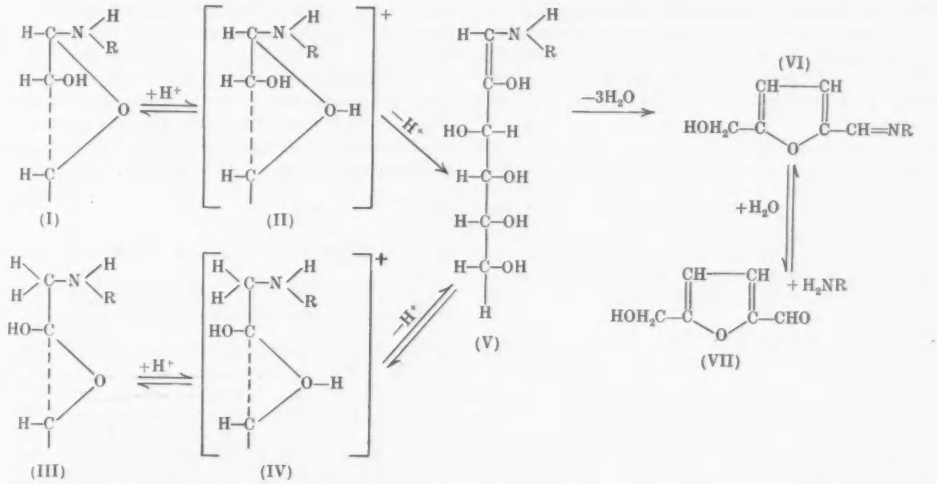
Experiment 5. Solutions containing approx. 5% (w/v) fructose-phenylalanine and 1% (w/v) D-glucose respectively in water and adjusted to pH 11·0 with alkali were heated at 100° for 10 min. and after neutralization run on paper. Only in the glucose experiment was glucose detectable; the other solution contained free phenylalanine and some residual fructosamine.

Experiment 6. A 0·1N-Na_2CO_3 solution containing 0·36% (w/v) *N-p*-tolylfructosamine was heated for 20 min. at 100° and after neutralization tested chromatographically; there was no residual fructosamine and a practically complete recovery of the liberated base.

DISCUSSION

It would appear that the compound formed in hot methanol from D-glucose and DL-phenylalanine can be identified as fructose-phenylalanine (*N*-substituted fructosamine) by its strong reducing power (*o*-dinitrobenzene in the cold), the fact that it cannot be hydrolysed by acid or alkali, by its colour reaction with *p*-DAB after pretreatment with acetylacetone and by its conversion to 5-hydroxymethylfurfuraldehyde and phenylalanine; no other structure would fit these properties. The inability of the compound to be hydrolysed by acid contrasts with the ready hydrolysis by weak acids of the *N*-glucosylamino-acids prepared by Micheel & Klemer (1951) and by Pigman, Cleveland, Couch & Cleveland (1951). The reduction in the cold of *o*-dinitrobenzene was found by Kuhn & Weygand (1937) to be a characteristic property of fructosamines distinguishing them from the isomeric *N*-substituted glucosylamines. The colour reaction

with *p*-DAB is known to be given only by sugars with an amino group attached to $C_{(1)}$ or $C_{(2)}$ (Elson & Morgan, 1933). The ready transformation to 5-hydroxymethylfurfuraldehyde of the compound by acid is incompatible with the displacement by an NHR group of the hydroxyl group at $C_{(2)}$ since this hydroxyl group is essential for the conversion of a hexose to 5-hydroxymethylfurfuraldehyde; thus D-glucosamine cannot be converted to the furfuraldehyde derivative even by strong mineral acid. In our compound, therefore, the amino group must be linked to $C_{(1)}$ and there must be a free reducing group at $C_{(2)}$ because the compound, though not hydrolysable, is strongly reducing.



Fructose-phenylalanine shows no tendency to crystallize, a property which it shares with the *N*-glucosylamino-acids and with a number of *N*-arylfructosamines which were found previously to be uncrystallizable (Micheel & Klemer, 1951; Weygand, 1940).

The outstanding feature of the *N*-substituted fructosamines (III) investigated, the crystalline *N*-*p*-tolyl-D-fructosamine and the amorphous phenylalanine analogue, is the ease with which in the presence of weak acid they rearrange and give off water to form the Schiff's base (VI) of 5-hydroxymethylfurfuraldehyde (VII) which in turn is hydrolysed to VII and the free amine. The degradation of (III) to (VII) has a pH optimum; it proceeds more quickly with 2N-acetic acid than with N-hydrochloric acid. Presumably the reaction involves a preliminary acid-base equilibrium yielding the conjugate acid (IV) followed by transformation of (IV) into an activated complex (of unknown structure) which by loss of a proton changes reversibly to the enol form (V). It is of interest that *N*-*p*-tolylglucosylamine (I), when quickly dissolved (otherwise hydrolysis prevails) and heated in 2N-acetic acid, is also largely degraded to (VII), the concomitant hydrolysis to glucose and base playing a minor role. As indicated in the diagram the conversion of the secondary *N*-glucosylamine (I) into the enol (V) must be assumed to be irreversible; whereas *N*-arylglucosylamines are convertible to the corresponding fructosamines, the reverse reaction has not been observed; the same seems to hold for the *N*-glucosylamino-acids.

The facility with which compounds of the general structure (I) and (III) produce (VII) on mild acid treatment is conditioned (*a*) by the basicity of the group NHR thus favouring the formation of the respective conjugate acids (II) and (IV) and in turn of the enol form (V), and (*b*) by the presence in the enol form of the tautomeric mobile vinylamine structure. D-Fructose, lacking the basic N atom at $C_{(1)}$, did not yield (VII) in appreciable amounts under conditions where fructose-phenylalanine formed (VII) up to 63% of theory; yet of the hexoses D-fructose is the sugar most sensitive to acid probably owing to the presence in its solution of considerable quantities of the furanose form (Gottschalk, 1943). Compound (VII) is the main product of the degradation of fructose-phenylalanine when treated with 2N-acetic acid. Its ready formation under such mild conditions reflects the marked effect of the replacement of the primary hydroxyl group at $C_{(1)}$ of a ketohexose by the basic NHR group on the reactivity of the sugar molecule. A useful tool is thus provided for the detection of the

fructosamine structure in natural products like mucoproteins and mucopolysaccharides.

The observation by Akabori (1933) that amino-acids when heated with furfuraldehyde or with (VII) undergo oxidative deamination and decarboxylation may account for the incomplete recovery of the amino-acid in an experiment in which fructose-phenylalanine was treated with acetic acid. With *p*-toluidine as the amine the loss in (VII) and in *p*-toluidine upon acetic acid treatment of the parent compounds (I) and (III) is explained by the presence among the reaction products of the Schiff's base (VI) and of considerable amounts of insoluble humin (Table 1, Fig. 1). When treated with N-HCl the *N*-substituted fructosamines yield only small quantities of (VII) due to secondary reactions of (VII) induced by the mineral acid (Exp. 3).

N-Substituted fructosamines just like glucosamine and glucosylamine form a deep colour with *p*-DAB only after pretreatment with acetylacetone; the identity of the absorption bands of the coloured compounds from glucosamine and the *N*-alkyl-fructosamine suggests closely related chromophoric structures. Most probably, as proposed by Elson & Morgan (1933), these chromophores are pyrrol derivatives.

SUMMARY

1. The *N*-substituted fructosamine (*iso*glucosamine) fructose-phenylalanine (*N*-(1′-carboxy-2′-phenylethyl)amino-1-deoxyfructose) has been prepared. The product, which showed no tendency to crystallize, was not completely free of impurities; the contaminants, however, were present in small amounts only.

2. The compound's characteristic reactions were also given by *N*-*p*-tolyl-D-fructosamine, a crystalline *N*-arylfructosamine. The outstanding property of *N*-substituted fructosamines tested is the ease with which upon mild acid treatment (2N-acetic acid) they form 5-hydroxymethylfurfuraldehyde, in the case of the phenylalanine compound up to 63 % of the theory.

3. Since under the same conditions even the acid-labile D-fructose does not yield appreciable amounts of 5-hydroxymethylfurfuraldehyde, its production by dilute acetic acid may be used as a tracer for *N*-substituted fructosamines in natural products.

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Studies of Sebum

3. METHODS FOR THE COLLECTION AND ESTIMATION OF SMALL AMOUNTS OF SEBUM

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In the course of investigations into the nature and functions of sebum it was necessary to measure the 'sebum levels' (i.e. the amount of sebum which covers 1 sq.cm. of skin) and rates of secretion of different areas of the human body. This necessitated the collection of samples of sebum from an accurately measured area of skin and the estimation of the quantity of sebum thus obtained. The methods that have been used for the collection of sebum have been reviewed elsewhere (Wheatley, 1952); of these only one can be used for the quantitative collection of sebum from a measured area of skin. This method entails the extraction with a fat solvent of an area of skin demarcated by means of a cup or cylinder held against the skin by manual pressure, or by suction applied to an outer chamber, and has

already been widely used with satisfactory results. The fat solvent used differs widely, but ether is the most popular, and is used either alone (Emanuel, 1936; Kvorning, 1949) or mixed with ethanol (Butcher & Parnell, 1947) or chloroform (Carrié, 1936).

The methods used for the subsequent estimation of the sebum thus collected are, however, open to criticism. Emanuel (1936) and Butcher & Parnell (1947) have used a nephelometric method. Chromate oxidation methods have been used and vary from the original Bang method (Carrié & Neuhaus, 1951) to the more elegant manometric method of Kirk, Page & Van Slyke (1934) used by Kvorning (1949); while a fatty acid titration method has been used by Carrié & Ottofrickenstein (1942). In no case has the method been standardized with samples of sebum; instead, either an arbitrary standard was used or an indirect calculation made. While the present investigation was in progress Herrmann & Prose (1951) drew attention to the inaccuracies of these procedures and advocated direct weighing of the sebum sample. A more detailed study has here been made of the methods available, and their reliability assessed by comparison with direct weighing.

MATERIALS AND METHODS

Collection of sebum. The method adopted was almost identical with that used by Kvorning (1949). A glass cylinder (generally of 3 cm. diam.) was pressed firmly against the skin. Fat-free solvent (5 ml., previously twice redistilled in all-glass apparatus), was pipetted into this cylinder and allowed to remain in contact with the skin for 60 sec. At the end of this period the solvent was transferred to a clean test tube by means of a teat pipette, the cylinder and area of skin were then washed with a further 3 ml. of solvent and the washings added to the first extract. The solution of sebum, which also contained cellular debris and dirt, was filtered through a sintered-glass microfilter and was then ready for the subsequent estimation. CCl_4 was selected as the most suitable solvent after preliminary trials with acetone, ether and $CHCl_3$.

Methods of estimation. (*a*) *Gravimetric method.* After filtration the solution of sebum in CCl_4 was evaporated to dryness in a weighed 10 ml. conical microflask and the flask and its contents were dried *in vacuo* over conc. H_2SO_4 for 2 hr. or more and then reweighed on a micro- or semimicro-balance. (A blank determination should be made with each series of weighings.) A determination was then made on the residue of sebum by one of the following methods.

(*b*) *Acid-number method.* The residue of sebum was dissolved in 2 ml. $CHCl_3$ and the solution titrated with 0·005 N-NaOH using cresol red as indicator and stirring by means of a stream of N_2 (cf. Moyle, Baldwin & Scarisbrick, 1948).

(*c*) *Iodine-number method.* A modification of the method of Yasuda (1931) was used. The sebum was dissolved in 2–5 ml. CCl_4, 1 ml. 0·04 N-pyridine dibromide reagent (freshly made by diluting a 0·1 N stock solution with glacial acetic acid) was then added, the mixture shaken and allowed to stand for 15 min. At the end of this period 1 ml. 3% KI solution was then added and the liberated I_2 titrated with 0·005 N-$Na_2S_2O_3$ solution.

(*d*) *Chromate-oxidation method.* The method of Bloor (1928, 1947) was used without modification. The silver dichromate reagent (3 or 5 ml.) was added to the microflask: this was then placed in a boiling-water bath for 15 min. It was then removed from the bath, 3 ml. water were carefully added and the flask was allowed to stand for a further 10 min. to cool. The contents were then transferred to a 175 ml. conical flask with 75 ml. water: 1 ml. 10% KI solution was then added and the liberated I_2 titrated with 0·1 N-$Na_2S_2O_3$ solution.

(*e*) *Turbidimetric method.* The method used by Butcher & Parnell (1947) was adapted for an ordinary test tube type of photoelectric colorimeter. The sebum was saponified by heating in a water bath with 1 ml. 1% ethanolic KOH, the solution was carefully evaporated almost to dryness, diluted to 7 ml. with water and then acidified by the addition of 0·2 ml. conc. HCl. The mixture was then thoroughly shaken and the turbidity read in an EEL photoelectric colorimeter (Evans Electroselenium Ltd.) using an Ilford no. 608 spectrum-red filter. The optical density thus obtained refers to a thickness of solution of approx. 1·4 cm.

RESULTS

The use of a simple glass cylinder, as advocated by Kvorning (1949), was found to be entirely satisfactory as a method of collecting sebum and, provided that sufficient care was taken, the use of suction device (as recommended by Emanuel, 1936) to keep this cylinder pressed against the skin was found unnecessary. Earlier work (MacKenna, Wheatley & Wormall, 1950) has shown that sebum is only slightly soluble in ethanol and acetone, rather more soluble in ether, and readily soluble in chloroform and carbon tetrachloride. The last two solvents appeared to be the most suitable for use in the cylinder, and of these carbon tetrachloride is more inert and is less irritant to the skin. Ether proved particularly unsuitable; not only does it require to be kept peroxide-free, but it also was found to remove significant amounts of non-lipid material from the skin. In contradistinction to the findings of Herrmann & Prose (1951) it was found necessary to filter the sebum solution before proceeding with the analysis, for with some samples the amount of horny-layer scales removed by filtration would have apparently raised the sebum level by as much as 30 μg./sq.cm.

Samples of sebum were collected from various areas of the body from a number of normal subjects and also from patients with certain skin diseases. The sample was first weighed, then an estimation was performed by one of the other methods described; a random selection of sebum samples was studied by each method. Dot diagrams were drawn in which the weight of sebum was plotted against the titration figure or colorimeter reading, and the reliability of each method assessed. The following results were obtained.

Acid-number method. Thirty samples were estimated by this method with the results shown in Fig. 1. It was found that 1 mg. of sebum was equivalent to $0{\cdot}163 \pm 0{\cdot}083$ ml. of 0·005 N-NaOH. These results show that the acid number of sebum varies widely and that this method cannot satisfactorily be used to estimate sebum. The amount of alkali required in the titrations was very small and for this reason accurate results were not possible. In view of these discouraging results it was not considered worth while to investigate a method in which the total fatty acids liberated after saponification were titrated.

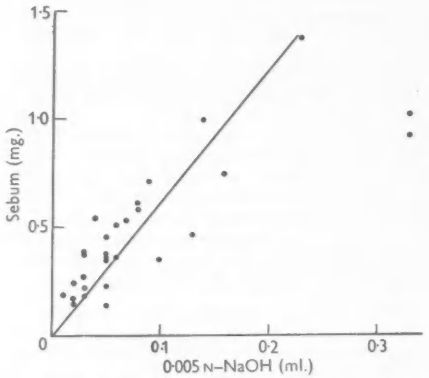

Fig. 1. Attempt to estimate sebum by direct titration with alkali (acid-number method).

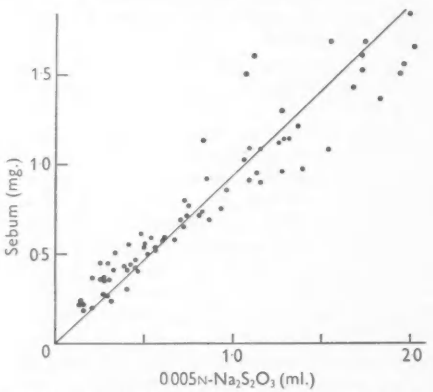

Fig. 2. Results obtained when the iodine uptake of sebum is used as a method of estimation (iodine-number method).

Iodine-number method. The results of seventy-five determinations by this method are shown in Fig. 2. With this method it was found that 1 mg. sebum $\equiv 1{\cdot}06 \pm 0{\cdot}22$ ml. of 0·005 N-$Na_2S_2O_3$, and the method had an average error of ± 15·8 %. The iodine number of sebum appears to vary widely and results obtained in the present method indicate a range of 35–90, with an average of 67. It was found, moreover, that the results from one area of the body (namely the forearm) showed the same range of variation as did those from all areas of the body; this suggests that the variations are due to differences in the composition of sebum from person to person rather than differences in composition over the surface of the body. The method appears, therefore, to have some uses in the study of a given subject after a calibration has first been made with samples of sebum from the person concerned.

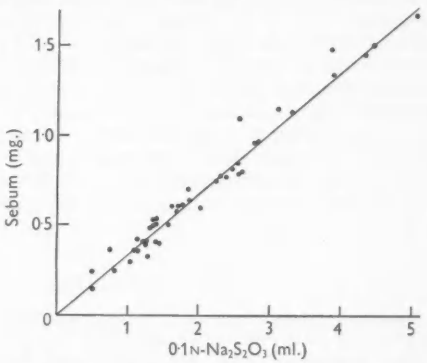

Fig. 3. Calibration curve for the estimation of sebum by the chromate-oxidation method.

Chromate-oxidation method. Estimations were made on forty-five samples of sebum by this method with results as shown in Fig. 3. The results gave a calibration factor for sebum of 1 mg. $\equiv 2{\cdot}94 \pm 0{\cdot}33$ ml. of 0·1 N-$Na_2S_2O_3$, and indicated that the method was very sensitive and had an average error of ± 9·0 %. A similar calibration value was obtained with samples of sebum collected from the whole forearm by the method described by MacKenna *et al.* (1950). When cholesterol was oxidized under the conditions of analysis described here, a factor of 1 mg. $\equiv 3{\cdot}85$ ml. of 0·1 N-$Na_2S_2O_3$ was obtained, compared with the theoretical value of 3·92 ml. (Bloor, 1928). This indicates that oxidation was practically complete, but the results obtained with sebum indicate an apparent carbon content of 64 %, compared with 77 % for human plasma lipids (Kirk *et al.* 1934).

Turbidimetic method. The results of forty-five estimations by this method are shown in Fig. 4. The calibration curve is not linear, an observation which has been confirmed by performing a series of estimations with different weights of the same sample of forearm sebum. The method has an average error of

$\pm 11{\cdot}6\%$; as described here it is not very sensitive, but the sensitivity can be increased by using a nephelometer instead of an ordinary photoelectric colorimeter. The average turbidity produced by sebum was found to be 102% of that produced by triolein under these conditions of analysis.

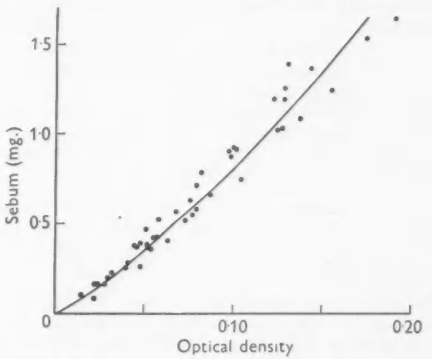

Fig. 4. Calibration curve for the turbidimetric estimation of sebum.

Gravimetric method. The weight of sebum collected has been used as standard against which the results obtained by the other methods have been compared. Such a gravimetric method is not completely free from error, even excluding weighing errors, since it is assumed that all the material in the carbon tetrachloride solution is lipid. Under certain conditions samples may be contaminated by such substances as urea and lactic acid which originate from sweat. Such contamination is probably negligible under most conditions of collection, and it is thought unlikely that the criticism applies to the results presented here.

The results of estimations of the sebum levels of a number of different areas of the body, obtained by the gravimetric method, are shown in Table 1. These results are in general agreement with those obtained by other workers and confirm the finding that the sebum level is highest in the scalp, decreases over the body, and is lowest in the extremities.

DISCUSSION

Of the methods available for the estimation of small amounts of sebum, direct weighing of the sample appears to be the method of choice; as this is tedious and requires a more sensitive balance than the ordinary analytical type, such a method cannot always be used. Alternative methods are turbidimetric (or nephelometric) estimation, and estimation of the total organic matter by chromate oxidation. Both of these methods give results that are sufficiently accurate for most purposes, but it is desirable that the method to be used should first be calibrated using samples of pure sebum. Samples large enough for such calibration can easily be collected from the forearm by the method described in the preceding paper of this series (MacKenna, Wheatley & Wormall, 1952). Where such a calibration is not practicable the following recommendations are made. (*a*) In the turbidimetric method triolein can conveniently be used as a standard since it has been found to produce practically the same turbidity as sebum. (*b*) Provided that the conditions of oxidation described here are adhered to, a factor of 2·94 ml. of 0·1 N-$K_2Cr_2O_7$/mg. sebum can be used for the chromate-oxidation method. This factor corresponds to a carbon content of only 64% and it is suggested that under our conditions of analysis the hydrocarbon fraction of sebum is not oxidized. This does not occur in all chromate-oxidation methods, for when the more drastic conditions of the Kirk, Page & Van Slyke (1934) method are used hydrocarbons appear to be satisfactorily oxidized (cf. Kvorning, 1950). It is therefore necessary to calibrate chromate methods using slightly different conditions of oxidation from those described here.

After the present studies had been completed a paper by Jones, Spencer & Sanchez (1951) appeared, in which the authors describe a very sensitive surface film method of estimating sebum. We hope to make a more detailed study of this method which will, no

Table 1. *Some sebum levels of various sites of the body obtained by the gravimetric method of estimation*

Site of body	No. of subjects	No. of estimations	Sebum level (μg./sq.cm.) Range	Average
Forehead	17	22	97–340	212±73
Chest	21	24	44–237	120±61
Back	27	40	21–268	106±56
Abdomen	13	41	25–227	67±45
Axilla	8	12	30–237	84±59
Arm	38	63	9–146	58±34
Groin	4	4	50–105	75±28
Leg	11	13	18–82	36±19

doubt, prove very useful in certain investigations owing to its high degree of sensitivity.

The wide variations of both the acid number and the iodine number indicate variations in the composition of sebum. Squalene is the most unsaturated constituent of sebum and small changes in its concentration would produce wide fluctuation in the iodine number. This and other variations in the composition of sebum are now being studied in order to obtain further information about certain skin diseases.

SUMMARY

1. Methods available for the collection and estimation of sebum have been studied in order to assess their reliability.

2. The cup method of collection has been found satisfactory when carbon tetrachloride is used as solvent.

3. Direct weighing of the sebum sample is recommended, but reliable results can be obtained with either the nephelometric or the chromate-oxidation method provided that the method has previously been calibrated by means of samples of sebum.

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The Preparation and Properties of β-Glucuronidase

4. INHIBITION BY SUGAR ACIDS AND THEIR LACTONES

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Karunairatnam & Levvy (1949) showed that animal β-glucuronidase was strongly inhibited by saccharate and feebly by mucate solutions. These observations were confirmed by Mills & Paul (1949), who claimed greater inhibition by saccharate than had been obtained by Karunairatnam & Levvy. Spencer & Williams (1951) found, on the contrary, that the action of saccharate was weaker than had originally been stated. Campbell (1949), in a histochemical study of β-glucuronidase, found that the enzyme in frozen mouse-kidney sections was almost completely inhibited by 10^{-3}M-saccharate.

Other enzyme systems have been compared with β-glucuronidase on the basis of their response to the presence of saccharate. Thus, Karunairatnam & Levvy (1949) found that the glucuronide-synthesizing system in mouse-liver slices was not appreciably affected by saccharate. This finding was put forward in support of the view that β-glucuronidase is not involved in the biosynthesis of glucuronides. Satisfactory evidence to exclude the possibility that the inhibitory factor did not penetrate the intact cell was not available at that time, but has since been obtained by Campbell (1949) in his histochemical work, and by Karunairatnam (1950), who showed that hydrolysis of phenolphthalein glucuronide by mouse-liver slices was completely inhibited by 10^{-2}M-saccharate.

In their study of the glucuronide-decomposing enzyme obtained from the medium-sized micro-organisms in the rumen of the sheep, Karunairatnam & Levvy (1951) observed only 8% inhibition of the hydrolysis of $1{\cdot}25 \times 10^{-3}$M-phenolphthalein glucuronide by 10^{-2}M-saccharate, a concentration which caused complete inhibition of mouse-liver β-glucuronidase. However, Smith & Mills (1950) obtained marked inhibition of the glucuronide-decomposing enzyme in *Escherichia coli* with 10^{-3}M-saccharate, and Robinson, Smith & Williams (1952) reported that the similar enzyme in locust saliva was also inhibited by saccharate.

Saccharate given in large doses to mice failed to produce any physiological effects, such as might have been expected to result from arrest of β-glucuronidase action (Karunairatnam & Levvy, 1949; see also Carr, 1947). It was considered that saccharate may be rapidly metabolized *in vivo*, and that therefore a study should be made of 3-methylsaccharate as a possible inhibitor for β-glucuronidase which might be stable in the body. 3-Methylsaccharo-1:4-lactone was prepared and converted in solution into 3-methylsaccharate by treatment with alkali. On testing for inhibition of β-glucuronidase *in vitro*, the surprising result was obtained that the 1:4-lactone was quite a good inhibitor whilst 3-methylsaccharate itself was inactive. This at once suggested the possibility that inhibition by saccharate is in fact due to saccharo-1:4-lactone present as impurity in the solution. When a solution of saccharic acid is evaporated, a solid separates which consists largely of saccharo-3:6-lactone, but which also contains a little saccharo-1:4-lactone. The two lactones can be separated by fractional crystallization (Smith, 1944). These compounds have been studied for their effects on β-glucuronidase, and in the light of the results obtained the inhibitory actions of saccharate and mucate solutions have been reinvestigated and compared with those of other sugar acids.

EXPERIMENTAL

Materials. Potassium hydrogen saccharate, mucic acid and sodium gluconate were obtained from British Drug Houses Ltd., ascorbic acid from Light and Co. Ltd., and α-D-galacturonic acid from both Roche Products Ltd., and the Eastman Kodak Co. Glucurone was presented by the Corn Products Refining Co., and it was converted in solution into sodium glucuronate by treatment with NaOH. The diacetone derivative of 2-keto-L-gulonic acid was presented by Dr F. Bergel of Roche Products Ltd. Before use, a 10% (w/v) aqueous solution was boiled for 30 min. to remove the acetone groups (Reichstein & Gruessner, 1934). The residual solution was assumed to contain only 2-keto-L-gulonic acid. Mannurone and 3-methylglucuronic acid were prepared in this Institute by Mr C. A. Marsh. The former compound was converted in solution into sodium mannuronate by treatment with NaOH. 3-Methylsaccharo-1:4-lactone (Levene & Meyer, 1922) was converted in solution into sodium 3-methylsaccharate by treatment with NaOH. Saccharo-3:6-lactone was prepared as described by Smith (1944). Saccharo-1:4-lactone, saccharo-1:5-3:6-dilactone, mannosaccharo-1:4-3:6-dilactone and the amide of methyl 4-methylglucuronide were gifts from Prof. F. Smith, Department of Agriculture, University of Minnesota. Sodium mannosaccharate was obtained in solution by treatment of mannosaccharodilactone with NaOH. Methyl 4-methylglucuronide was obtained in solution from the amide by heating the latter with NaOH (Smith, 1951). The solution was then made N with respect to HCl and boiled for 3 hr. to split the glycoside link. Complete conversion of the starting material into 4-methylglucuronic acid was assumed.

The opening of lactone rings by treatment with NaOH was in every case controlled with the glass electrode. Alkali was added to bring the initial pH to 9 or 10. More alkali was added as the pH fell, until it became stable at an alkaline figure.

Enzyme preparation. The enzyme was prepared fresh daily from adult mouse liver. The liver was homogenized in water and made up to a final volume of 9 ml. After adding 1 ml. M-acetate, pH 5·2, the homogenate was incubated at 37° for 30 min., and centrifuged at 1500 *g* for 15 min. The supernatant was decanted and to it was added an equal volume of saturated $(NH_4)_2SO_4$ solution. Precipitated protein was separated by centrifuging at 1500 *g* for 15 min., and taken up in water. The final volume was usually adjusted to 25 ml.

Enzyme assay. To 1 ml. 0·5M-acetate, pH 5·2, and 0·5 ml. phenolphthalein glucuronide solution (Talalay, Fishman & Huggins, 1946) in a 10 or 15 ml. centrifuge tube were added 2 ml. water or inhibitor solution. The tube was immediately placed in a bath at 37°, and 0·5 ml. of the enzyme preparation was run in. After 1 hr., 4 ml. glycine-Na_2CO_3 buffer (Karunairatnam & Levvy, 1951) were added and the mixture was centrifuged at 1500 *g* for 15 min. The intensity of the red colour in the supernatant was read with a Spekker photoelectric absorptiometer, using Ilford no. 605 yellow-green filter. Except in measurements of K_i and K_m, the concentration of the phenolphthalein glucuronide solution was 0·005M, giving a final concentration in the incubation mixture of 0·00063M. In finding K_i and K_m, series of tubes were used in which the final substrate concentration ranged from 0·00125 to 0·000031M.

Before being added to the incubation mixture, inhibitor solutions were, if necessary, adjusted to pH 5·2 with HCl or NaOH (KOH in the case of potassium hydrogen saccharate), and suitably diluted. Wherever possible, pH adjustment was omitted with lactone solutions to be tested as such. But, if adjustment of pH was essential, it was carried out with great care immediately before addition to the assay tubes. When changes in the inhibitory power of a given solution were being measured at different time intervals, the experiments were so arranged that all samples of the solution came forward for test simultaneously on a single enzyme preparation.

RESULTS

Measurements of inhibitory power. Representative figures for the inhibition of glucuronidase by all the compounds examined are listed in Table 1. The effects of varying the concentrations of the more

powerful inhibitors are illustrated in Fig. 1. It is clear that the degree of inhibition observed with any given concentration of saccharate depended on the past history of the inhibitor solution. When the pH was adjusted to 5–5·2 to correspond to the pH of the enzyme-substrate mixture, the inhibitory power of the solution increased up to sixfold during the first few days at room temperature. The saccharate concentration at this stage, as opposed to the final concentration in the incubation with enzyme and substrate, could be varied widely with little, if any, change in the percentage increase in inhibitory power after a given period. Initial saccharate concentrations were therefore chosen entirely on grounds of convenience.

A more dramatic increase in inhibition by saccharate was observed when slightly acid solutions were boiled for a short time. The pH of freshly prepared solutions of potassium hydrogen saccharate varied from 3·7 at 0·05 M to 4·2 at 0·0005 M. In the particular experiments under discussion, 1 equivalent of hydrochloric acid was added to solutions of potassium hydrogen saccharate in the lower concentration range to give a pH of 3·5 before boiling. Wide variation in saccharate concentration made no difference to the increase in inhibitory power produced by boiling, nor, as shown later, did omission of the hydrochloric acid.

Saccharo-1:4-lactone was about three times more powerful an inhibitor than a boiled solution of saccharate, and was the most powerful inhibitor encountered. The other two lactones of saccharic acid which were studied were relatively feeble inhibitors, the 1:5-3:6-dilactone being about as

Table 1. *Effects of various sugar acids and their lactones on the hydrolysis of* 0·00063 M-*phenolphthalein glucuronide by mouse-liver glucuronidase*

(Unless stated to the contrary, inhibitor solutions were freshly prepared before use.)

Compound	Final concentration (10^{-6} M)	Inhibition (%)
Saccharate	1 800	50*
Saccharate (after 4 days at room temp. and pH 5)	300	50*
Saccharate (after boiling at pH 3·5 for 30 min.)	12	50*
Saccharo-1:4-lactone	3·6	50*
Saccharo-3:6-lactone	3 000	50*
Saccharo-1:5-3:6-dilactone	1 000	35
3-Methylsaccharate	10 000	0
3-Methylsaccharo-1:4-lactone	1 600	50*
Mucate	5 000	6
Mucate (after 1 day at room temp. and pH 5)	5 000	20
Mucate (after boiling at pH 2 for 1 hr.)	725	50*
Mannosaccharate	1 000	0
Mannosaccharo-1:4-3:6-dilactone	1 000	0
Glucuronate	10 000	52
Glucurone	10 000	0
3-Methylglucuronate	10 000	0
4-Methylglucuronate	1 000	0
Gluconate	10 000	0
Gluconate (after boiling at pH 3 for 4 hr.)	5 000	7
Galacturonate	10 000	14
Galacturonate (after 5 days at room temp. and pH 5)	10 000	24
Mannuronate	10 000	0
Mannurone	10 000	0
Ascorbate	1 000	0
2-Keto-L-gulonate	10 000	0

* Result read from Fig. 1.

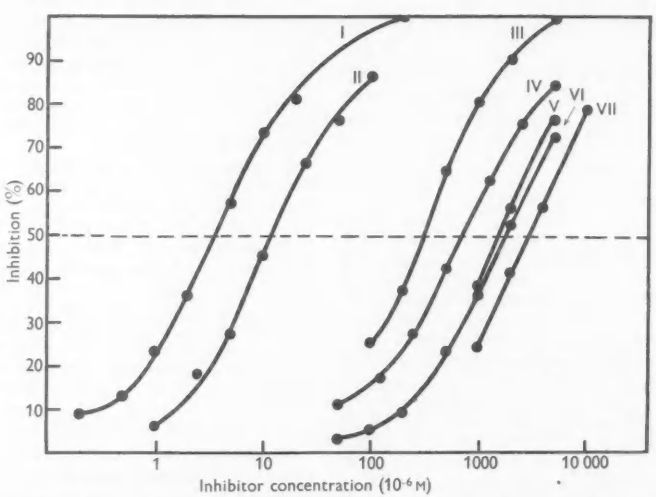


Fig. 1. Inhibition of the hydrolysis of 0·00063 M-phenolphthalein glucuronide by varying concentrations of: I, saccharo-1:4-lactone; II, saccharate boiled for 30 min. at pH 3·5; III, saccharate after standing for 4 days at pH 5; IV, mucate boiled for 1 hr. at pH 2; V, 3-methylsaccharo-1:4-lactone; VI, freshly prepared saccharate solution; VII, saccharo-3:6-lactone.

effective as freshly prepared saccharate solution and the 3:6-lactone somewhat weaker. Introduction of a methyl group into the hydroxyl at position 3 in the saccharate molecule abolished inhibition entirely, and considerably reduced the inhibitory power of the 1:4-lactone.

Table 2. *Apparent affinities of various inhibitors for mouse-liver β-glucuronidase compared with that of phenolphthalein glucuronide*

(Unless shown to the contrary, inhibitor solutions were freshly prepared before use.)

Compound	Apparent dissociation constant (10^{-6} M)	Apparent relative affinity
Phenolphthalein glucuronide	130±8 (19)*	1
Saccharate	170	0·8
Saccharate (after 2 days at room temp. and pH 5)	35	3·7
Saccharate (after boiling at pH 3·5 for 30 min.)	1·6	81
Saccharo-1:4-lactone	0·54	240
Saccharo-3:6-lactone	480	0·3
3-Methylsaccharo-1:4-lactone	130	1
Mucate	6000	0·02
Mucate (after boiling at pH 2 for 2 hr.)	89	1·5
Glucuronate	1600	0·08
Galacturonate	6000	0·02

* Mean±S.E. and, in parentheses, number of experiments.

Mucate (galactosaccharate) solutions displayed the same phenomena as saccharate (glucosaccharate) solutions, the inhibitory power increasing on standing at pH 5, and more strikingly so after boiling at an acid pH (in this instance pH 2, the pH of 0·01 M-mucic acid). Under all comparable conditions, however, mucate was much less effective than saccharate. Neither mannosaccharic acid nor its dilactone caused any inhibition of the enzyme.

Turning to the uronic acids listed in Table 1, it can be seen that the inhibitory power paralleled, on a much reduced scale, that of the corresponding saccharic acids, glucuronate being more powerful than galacturonate, and mannuronate quite inactive. Introduction of a methyl group into the hydroxyl at position 3 or 4 in the molecule abolished inhibition by glucuronic acid. Mannurone (mannurono-3:6-lactone) caused no inhibition of the enzyme, nor, in agreement with Spencer & Williams (1951), did glucurone (glucurono-3:6-lactone). Galacturonate solutions increased slightly in inhibitory power on standing at pH 5, perhaps due to a change to the β form from the α configuration of solid galacturonic acid.

The remaining compounds in Table 1 caused little, if any, inhibition of glucuronidase. Gluconic acid was quite inactive in freshly prepared solution. This compound can form 1:4- and 1:5-lactones, but a solution boiled at acid pH caused a barely perceptible degree of inhibition. Ascorbic acid was examined because of the report by Becker & Friedenwald (1949) that this compound inhibits rat-liver glucuronidase. In this laboratory, Karunairatnam (1950) confirmed this finding with mouse-liver glucuronidase, but considered ascorbate to be a relatively feeble inhibitor. In the

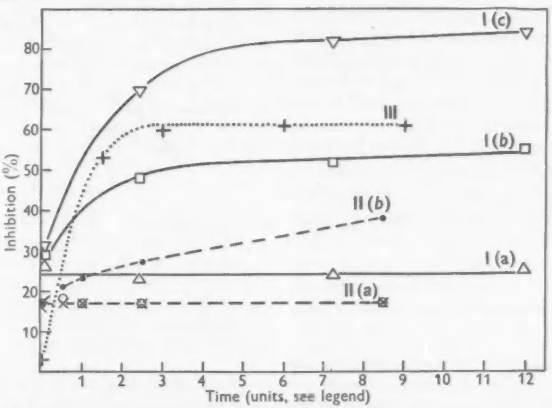


Fig. 2. Rate of increase in inhibitory power of saccharate solutions at varying temperature and pH (substrate 0·00063 M-phenolphthalein glucuronide). I, 5×10^{-4} M-saccharate solutions without added buffer, maintained at room temperature and pH (*a*) 7·4, (*b*) 5·2, (*c*) 4·2; time in units of 10 hr.; saccharate concentration in enzyme assay $2{\cdot}5 \times 10^{-4}$ M. II, 2×10^{-4} M-saccharate solutions in 10^{-3} M buffer maintained at 37° and pH (*a*) 7 and 6·1, (*b*) 5·2; time in units of 2 hr.; saccharate concentration in enzyme assay 10^{-4} M. III, 5×10^{-4} M-saccharate solutions without added buffer maintained at 100° and pH 4·2 or 3·5; time in units of 10 min.; saccharate concentration in enzyme assay $2{\cdot}5 \times 10^{-5}$ M.

present experiments, however, no inhibition whatsoever was observed with 0·001 M-ascorbate, whilst 0·01 M-ascorbate was found to interfere badly in the phenolphthalein colour reaction. Since ascorbic acid is a 1:4-lactone, the corresponding free acid, 2-keto-L-gulonic acid, was examined, again with negative results.

Values for K_i, the dissociation constant for the enzyme-inhibitor complex, were determined by the method of Lineweaver & Burk (1934) for nearly all the effective compounds, and the results are shown in Table 2. All these inhibitors acted competitively. Table 2 also gives a mean value for K_m, the dissociation constant for the enzyme-substrate complex, for phenolphthalein glucuronide, based on the values for all the enzyme preparations used in the K_i measurements. The mean value for K_m was taken as unity in calculating the relative affinities of the different inhibitors for the enzyme (affinity = 1/dissociation constant).

Inhibition by saccharate and mucate was clearly due, in large part at least, not to these ions as such, but to derivatives formed in aqueous solution. The term dissociation constant has no theoretical significance in such cases. Nevertheless, the apparent values for K_i give a useful measure of the inhibitory power of the different types of solution in a form which is independent of the nature and concentration of the substrate, and which permits comparison with figures obtained in earlier work.

The increase in inhibitory power of saccharate and mucate solutions with time

Fig. 2 shows the rate of increase in inhibitory power of saccharate solutions at varying temperature and pH. At room temperature, slightly acid solutions approached a limiting value in 2 or 3 days. This limiting value was somewhat lower when the pH of a potassium hydrogen saccharate solution, initially 4·2, was brought to 5·2 by addition of alkali. In saccharate solutions brought to neutrality the inhibitory power never increased beyond the initial low value. Other experiments, not illustrated, showed that addition of hydrochloric acid to potassium hydrogen saccharate solutions caused little change in the limiting inhibitory power approached at room temperature, but caused it to be reached more rapidly.

On boiling potassium hydrogen saccharate solutions, an equilibrium value for the inhibitory power was reached in 30 min. This value was the same and was reached at the same rate when 1 equiv. of HCl was added before boiling (final pH 3·5). The only effect of adding more HCl than this was to cause a falling off in inhibitory power when boiling was prolonged beyond 30 min. In experiments done at 37°, the pH values were chosen to correspond to those at which saccharate has been used in various enzyme studies, and the solutions were buffered with acetate to pH 5·2, and with phosphate to pH's 7 and 6·1. At pH 5·2, the inhibitory power still showed a steady increase when the experiment was terminated after 17 hr. At neither pH 7 nor 6·1 was there any increase in inhibitory power over the initial value throughout the period of incubation.

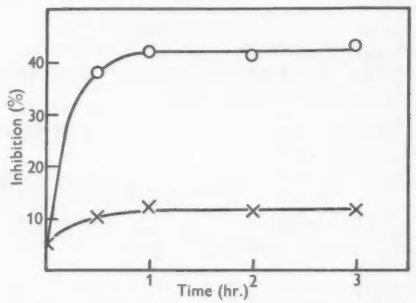


Fig. 3. Rate of increase in inhibitory power of 0·01 M-mucate solution on boiling at pH 2 (○—○) and 5·2 (×—×) in absence of added buffer (mucate concentration in enzyme assay 5×10^{-4} M; substrate 0·00063 M-phenolphthalein glucuronide).

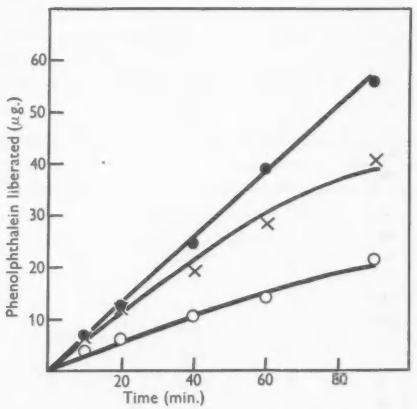


Fig. 4. The rate of hydrolysis of 0·00063 M-phenolphthalein glucuronide in presence and absence of saccharate. ●—●, no saccharate; ×—×, neutral saccharate solution, final concentration 5×10^{-4} M; ○—○, saccharate solution which had stood 5 days at room temperature at pH 5·2 in presence of 0·01 M-acetate buffer, final concentration $2{\cdot}5 \times 10^{-4}$ M.

Fig. 3 shows the rate of increase in inhibitory power of mucate solutions on boiling at the pH of 0·01 M-mucic acid and after adjustment to pH 5·2. The same process was apparently at work as in saccharate solutions.

The question arose whether the relatively small inhibition of glucuronidase caused by freshly prepared saccharate solutions, or by older solutions brought to neutrality, was due to saccharate ion itself, or whether it was due entirely to the more active molecule which is formed under the condi-

tions of enzyme assay (incubation at 37° and pH 5·2). Fig. 4 shows that the latter explanation was probably correct. The rate of hydrolysis of phenolphthalein glucuronide was at first unaltered by addition of a neutral solution of saccharate, but fell as incubation was prolonged. A saccharate solution which had stood for several days at room temperature and pH 5·2 slowed the hydrolysis of substrate straight away, although the results do suggest some increase in the percentage inhibition during the incubation, due no doubt to the effect of the rise in temperature on the formation of the inhibitor.

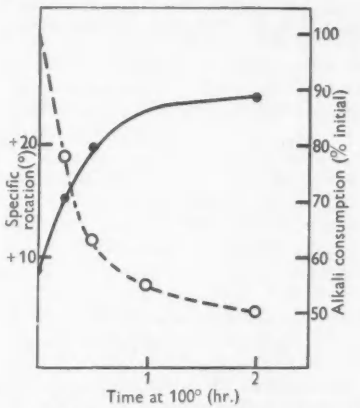


Fig. 5. Physical measurements of lactone formation in boiled solutions of potassium hydrogen saccharate. ●—●, $[\alpha]_D^{18°}$ (*c*, 1); ○- - -○, titration of 10^{-2}M-saccharate with 0·1N-KOH to pH 6 (glass electrode).

The derivatives of saccharic acid most likely to be formed in dilute aqueous solution are the 1:4- and 3:6-lactones. The latter is (Table 1) less effective an inhibitor for glucuronidase than a freshly prepared saccharate solution, whilst the former is very much more powerful. It seems plausible to suppose that saccharo-1:4-lactone formed in solution accounts entirely for the inhibitory action of saccharate. The increase in inhibitory power of a potassium hydrogen saccharate solution, with or without addition of HCl, reached a maximum after boiling for 30 min. (Fig. 2). The best measure of relative inhibitory powers is probably that given by the affinity figures (Table 2) and these suggest that one-third of the saccharate was converted into the 1:4-lactone after boiling for 30 min. Fig. 5 illustrates results obtained in attempts to follow lactone formation in boiled solutions of potassium hydrogen saccharate by polarimetric and titrimetric measurements. The changes measured are due, of course, to the formation of the 1:4-lactone, the 3:6-lactone, and perhaps also to the products of further molecular rearrangement (see Smith, 1944). The fall in alkali consumption corresponded to 37% lactonization after 30 min. at 100°, and 50% after 2 hr. Figures given in the literature for the rotation of saccharate solutions are rather variable. Our own figure for $[\alpha]_D^{18°}$ for a neutralized solution of potassium hydrogen saccharate was +9° (*c*, 1). Smith (1944) reports for $[\alpha]_D^{20°}$ for saccharo-1:4-lactone monohydrate and saccharo-3:6-lactone values of +34° and +45° respectively. Recalculating these for the difference in mol. wt. values of +29° and +35° are obtained for potassium hydrogen saccharate completely converted into the 1:4-lactone and 3:6-lactone respectively. The specific rotation of a saccharate solution in which one-third has been converted into the 1:4-lactone, but none into the 3:6-lactone, should be $(29+2\times9)/3=+15{\cdot}7°$. The value observed (Fig. 5) for a potassium hydrogen saccharate solution boiled for 30 min. was +19·7°. A solution to which had been added 1 equiv. of HCl gave a higher value (+25·2°).

The difficulties of interpretation associated with these measurements of lactone formation have been pointed out, but what the results do indicate is that molecular rearrangement in a saccharate solution can occur sufficiently rapidly on boiling to account for the inhibitory power in terms of the 1:4-lactone. Titrimetric measurements of lactonization in mucic acid solutions have been made by Taylor & Acree (1916), but lack of data for the pure lactones prevents comparison with the measurements of changes in the inhibitory power of mucate solutions.

The rate of change in inhibitory power of lactone solutions. Fig. 6 shows that a solution of saccharo-3:6-lactone, like saccharate solutions, increased in inhibitory power on standing at room temperature and pH 5, presumably due to transformation into the 1:4-lactone. The low degree of inhibition produced by a freshly prepared solution of the 3:6-lactone may have been due to the presence in the crystalline solid of a trace of the 1:4-lactone, or to formation of the latter in the solution under the conditions of enzyme assay. A velocity curve for the hydrolysis of phenolphthalein glucuronide in the presence of the 3:6-lactone (cf. Fig. 4) did not give a conclusive answer on this point. We have already seen that lactonization in a boiling solution of potassium hydrogen saccharate, as measured titrimetrically, continued after the inhibitory power of the solution had reached a maximum. This suggests that the 3:6-lactone, as such, does not inhibit glucuronidase. It can also be seen (Fig. 6) that decomposition of saccharo-1:4-lactone and 3-methylsaccharo-1:4-lactone, with consequent fall in inhibitory power, proceeded at comparable rates.

A point of some practical importance is the stability of saccharo-1:4-lactone (or of a boiled solution of potassium hydrogen saccharate) under the conditions in which it might be used in biochemical, physiological or microbiological experiments. Fig. 7 provides some information on this point. On incubating buffered solutions of the 1:4-lactone, pH 3·7–6·1, at 37° the percentage inhibition of the enzyme produced fell from 66 to about 53% in 16 hr. This represents a fall of rather less than half in the inhibitor concentration (cf. Fig. 1). Inhibition of the enzyme fell to 33% after the lactone solution had been incubated for 8 hr. at pH 7, corresponding to a fall of about 75% in inhibitor concentration. (Since the percentage inhibition of a given enzyme preparation is not a linear function of the inhibitor concentration, the latter cannot be directly deduced from the former. The inhibitory powers of two solutions are best compared by finding the relative amounts necessary to produce an identical effect on the enzyme. However, in that region in which the semi-logarithmic curves in Fig. 1 are approximately linear, they are roughly parallel, and doubling the inhibitor concentration corresponds to an increase of about 17% in the inhibition of the enzyme.)

The action of saccharo-1:4-lactone on other enzyme systems. As already mentioned, saccharate has been found to have no appreciable effect on the glucuronide-synthesizing system in mouse-liver slices (Karunairatnam & Levvy, 1949), and only

a slight inhibitory effect on the glucuronide-decomposing enzyme obtained from the medium-sized micro-organisms in the sheep rumen (Karunairatnam & Levvy, 1951). The enzyme assays in these two cases were carried out at neutrality and pH 6·1 respectively, conditions under which there can have been no transformation of saccharate into the 1:4-lactone (cf. Fig. 2). Nevertheless, a trace of the latter may have been formed in the original potassium hydrogen saccharate solution, and survived pH adjustment. The effects of saccharo-1:4-lactone on these two enzyme systems were therefore examined.

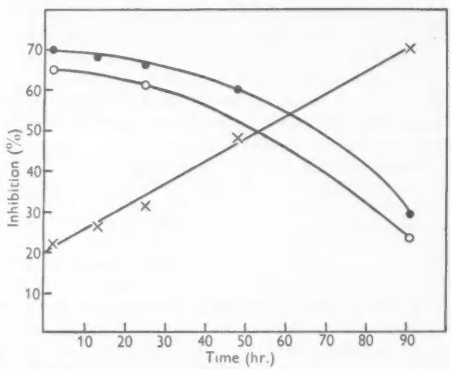


Fig. 6. The change in the inhibitory power of lactone solutions adjusted to pH 5, but without added buffer, on standing at room temperature (substrate 0·00063 M-phenolphthalein glucuronide; inhibitor concentrations halved in enzyme assay). ×—×, 2×10^{-3} M-saccharo-3:6-lactone; ●—●, 2×10^{-5} M-saccharo-1:4-lactone; ○—○, 4×10^{-3} M-3-methylsaccharo-1:4-lactone.

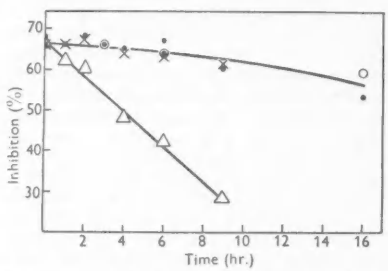
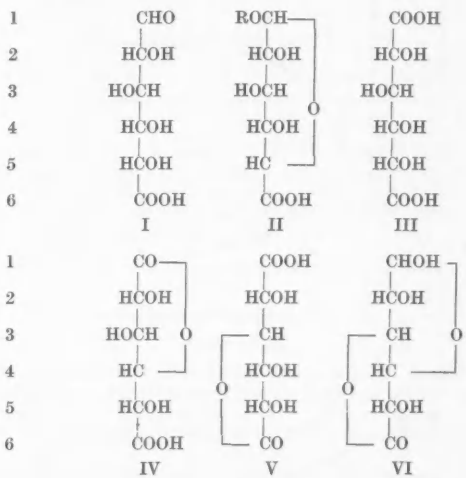


Fig. 7. The fall in the inhibitory power of 2×10^{-5} M-saccharo-1:4-lactone on incubating at 37° in 10^{-3} M buffer (substrate 0·00063 M-phenolphthalein glucuronide; inhibitor concentration halved in enzyme assay; pH's 7 and 6·1, phosphate buffer; pH's 5·2 and 3·7, acetate buffer). ●—●, pH 5·2; ×—×, pH 6·1; ○—○, pH 3·7; △—△, pH 7.

In a concentration of 0·0002 M, saccharo-1:4-lactone had no effect whatsoever on glucuronide synthesis by mouse-liver slices as measured by the method of Levvy & Storey (1949). Decomposition of the lactone during the 1 hr. incubation must have been negligible for present purposes (cf. Fig. 7). In a concentration of 0·00025 M, the 1:4-lactone caused 49% inhibition of the glucuronide-decomposing enzyme from the sheep rumen (substrate 0·0005 M-phenolphthalein glucuronide). The lactone is thus quite an efficient inhibitor for this enzyme, although the affinity is clearly much lower than in the case of β-glucuronidase.

DISCUSSION

From the evidence presented, it would appear to be safe to conclude that saccharo-1:4-lactone (IV) is the inhibitory factor for β-glucuronidase in saccharate solutions. This lactone had a very high



affinity for the enzyme, 240 times greater than that of phenolphthalein glucuronide, the substrate with the highest known affinity (cf. Karunairatnam & Levvy, 1949; Spencer & Williams, 1951), a finding which casts new light on the structural requirements for an efficient inhibitor of β-glucuronidase. The suggestion made by certain authors, for example Spencer & Williams (1951), that a strong inhibitor should possess two carboxyl groups is not supported by our new evidence.

Whilst the straight-chain formula for glucuronic acid (I) is resembled by the formula for saccharic acid (III), the general formula for a β-D-glucuronide (II) is more closely resembled by that for saccharo-1:4-lactone, which has a ring system involving $C_{(1)}$ and a single carboxyl group at $C_{(6)}$. The configuration of the secondary alcohol groups is of obvious importance since saccharo-3:6-lactone (V) was not an inhibitor. Passage from the saccharate to the mucate series by reversal of the configuration of $C_{(4)}$ resulted in a fall in inhibitory power, as did substitution in the hydroxyl at $C_{(3)}$. No inhibition at all was observed in the mannosaccharate series,

in which the configuration of the hydroxyl at $C_{(2)}$ in saccharate is reversed.

In accordance with previous work (Karunairatnam & Levvy, 1949; Spencer & Williams, 1951), glucuronic acid, one of the products of glucuronidase action, was found to cause some inhibition of the enzyme. Altering the configuration of the hydroxyl group at $C_{(4)}$ to give galacturonic acid reduced the inhibitory power, and it was entirely lacking in mannuronic acid. Spencer & Williams (1951) suggested that the failure of glucurone (VI) to inhibit glucuronidase was due, in part, to the presence of a 1:4-ring as opposed to the 1:5-ring in a glucuronide (II). In view of the action of saccharo-1:4-lactone it seems more probable that the loss of the carboxyl group at $C_{(6)}$ accounts entirely for the lack of inhibition by glucurone.

Since the saccharo-1:4-lactone content of a saccharate solution increases with the age of the solution to an extent dependent on the pH and temperature, and can further increase under the conditions of enzyme assay, previous measurements of the kinetics of inhibition of β-glucuronidase by saccharate have little meaning. The value of 6×10^{-5}M obtained by Karunairatnam & Levvy (1949) for K_i, the dissociation constant for the enzyme-inhibitor complex, approaches the figure of $3{\cdot}5 \times 10^{-5}$M given by Table 2 for the apparent dissociation constant for a saccharate solution which had stood some time at room temperature and pH 5. Spencer & Williams (1951) obtained a figure of 88×10^{-5}M for K_i for a neutral saccharate solution which contrasts with the figure of 17×10^{-5}M given by Table 2 for a freshly prepared saccharate solution. It would appear that less lactonization of saccharate occurred under the conditions of enzyme assay employed by Spencer & Williams than in those described above, but no explanation as to why this should have happened can be suggested. Mills & Paul (1949) have published no details of their measurements of the inhibitory action of saccharate.

The fact that the inhibition of glucuronidase observed with saccharate is an artifact arising from the acid conditions used for the enzyme assay could in itself explain the failure of saccharate to produce any effects in the living animal (Karunairatnam & Levvy, 1949). The instability of saccharo-1:4-lactone in neutral solution unfortunately limits its usefulness for physiological experiments, and the same is true of the 3-methyl derivative. Since saccharo-1:4-lactone is much more stable at slightly acid pH, it may prove useful in elucidating the function of glucuronide-decomposing enzymes in micro-organisms, in particular the enzyme in the rumen of the sheep. The difficulty of preparing the 1:4-lactone in any quantity need not be a disadvantage since the pure compound can be replaced by a solution of potassium hydrogen saccharate which has been boiled for 30 min., with resultant conversion of one-third of the saccharate into the 1:4-lactone.

Since the glucuronide-decomposing enzyme from the medium-sized micro-organisms in the rumen of the sheep (Karunairatnam & Levvy, 1951) showed marked inhibition in the presence of saccharo-1:4-lactone, this enzyme falls into line with all others with a similar action which have been tested for inhibition by saccharate. As pointed out above, no formation of the 1:4-lactone can have occurred at the pH of assay of the rumen enzyme, explaining the failure of freshly prepared saccharate solutions to cause marked inhibition. The failure of saccharo-1:4-lactone in relatively high concentration to affect glucuronide synthesis by mouse-liver slices confirms the view that β-glucuronidase does not participate in this process (Karunairatnam & Levvy, 1949; Karunairatnam, Kerr & Levvy, 1949).

SUMMARY

1. Saccharo-1:4-lactone is a very powerful inhibitor for β-glucuronidase, having an affinity for the enzyme 240 times greater than that of phenolphthalein glucuronide, the substrate with the highest known affinity. Inhibition is competitive. Saccharo-3:6-lactone causes little, if any, inhibition of the enzyme.

2. Saccharate ion as such does not inhibit β-glucuronidase. The inhibitory action reported by several workers is due to varying amounts of saccharo-1:4-lactone formed in acid solution before or during the incubation with the enzyme. Inhibition of the enzyme by mucate solutions is probably due to a similar cause.

3. One-third of the potassium hydrogen saccharate in an aqueous solution is converted into saccharo-1:4-lactone after boiling for 30 min.

4. The structural requirements for an efficient inhibitor of β-glucuronidase and other points arising from the above findings have been studied and are discussed.

The author wishes to express his gratitude to Prof. F. Smith for his generous gifts of material and valuable advice, and to those others mentioned in the text who presented materials, to Mr A. Hay for technical assistance, to Mr C. A. Marsh for help with many experiments, to Dr P. G. Walker for measurements of glucuronide synthesis and to Dr J. Conchie for polarimetric measurements.

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Incorporation of [*Carboxy*-^{14}C]Acetate into Lactose and Glycerol by the Lactating Goat Udder

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The ruminant udder is very active in the secretion of glycerides, and, since recent work has demonstrated that a large proportion of the milk fatty acids are synthesized from small molecules in the udder (Popják, French & Folley, 1951), the origin of the glyceride glycerol is of some interest. Experiments with slices of mammary glands have also shown that glycerol can be a limiting factor in fatty acid synthesis in mammary tissue (Balmain & Folley, 1951; Balmain, Folley & Glascock, 1952).

French & Popják (1951) obtained evidence of the conversion of glucose to glycerol in the mammary gland of the lactating rabbit. The specific activity of the glycerol isolated from the milk glycerides after administration of [^{14}C]glucose was very nearly the same as that of the lactose, and on the assumption that the latter value is a measure of the specific activity of the hexose available as substrate in the gland, it was calculated that within 6 hr. 50–100% of the glycerol was newly formed in the mamma.

Isolation of radioactive lactose from the milk secreted by the isolated bovine udder perfused with blood, containing [*carboxy*-^{14}C]acetate, has shown that the udder can incorporate acetate-carbon into lactose (Cowie *et al.* 1951). The glycerol of milk glycerides has subsequently been isolated from the experiment of Cowie *et al.* (1951), and its specific activity was found to be 10% of that of lactose, from which it follows that the udder can use acetate-carbon for glycerol formation via hexose, if not by a more direct route.

An experiment carried out by Popják *et al.* (1951) primarily with the object of studying the mechanism of the synthesis of the milk fatty acids, and in which [*carboxy*-^{14}C]acetate was injected intravenously into a lactating goat, provided an opportunity of obtaining further information about the origin of the glycerol of the milk glycerides. Lactose and glycerol have now been isolated from milk samples taken from this goat at frequent intervals after the injection, and their specific activities have been determined.

METHODS AND EXPERIMENTAL

The experimental procedure used has already been described (Popják *et al.* 1951). The amount of ^{14}C injected, as $CH_3{}^{14}COONa$, was 5 mc.

Isolation of lactose. The milk samples, from which nearly all the fat had been removed by centrifugation, were dried from the frozen state; they were stored in the cold-room for more than 2 years. Lactose was extracted from the milk samples with 70% (v/v) aqueous ethanol in a Soxhlet apparatus and was purified by recrystallization.

Isolation of glycerol. The glyceride fraction of the milk fat was saponified and, after removal of the fatty acids from the acidified hydrolysate by steam-distillation and by extraction with light petroleum (b.p. 40–60°), the aqueous phase

(also stored for more than 2 years) was neutralized with KOH and evaporated to dryness. The glycerol was extracted from the salts with boiling ethanol, the extract filtered and the ethanol removed by distillation under reduced pressure. Glycerol tribenzoate was prepared by benzoylation in water in an alkaline medium. After the benzoylated product had solidified (usually overnight at 4°), the glycerol tribenzoate was filtered off, washed with water, dried on a porous plate and crystallized twice from methanol.

Assay of ^{14}C. The radioactivity of glycerol tribenzoate was measured in 'infinite thickness' samples with an end-window Geiger-Müller counter (cf. Popják, 1950). Lactose was combusted in a stream of O_2 and the CO_2 collected, as described by Glascock (1951), and counted in an internal gas counter by the method of Brown & Miller (1947). In order to make the counts obtained by the two techniques comparable, a sample of the glycerol tribenzoate was also combusted and counted in the gas counter. A factor was thus obtained with which all the radioactive counts of the solid samples of glycerol tribenzoate were converted to the values of assaying the gas. Fortuitously, the radioactive counts obtained by assaying the solids had to be multiplied by a factor of only 0·99 to give the counts/mg. C as found by the gas-counting technique. The standard error of the radioactive counts of the samples up to the 12th hr. of the experiment was not more than 1%; the standard error of the counts beyond that time was 2–4%. The specific activities of both lactose and glycerol are expressed as counts/min./mg. C.

Degradation of glycerol. The periodate oxidation of glycerol was adapted for this purpose, the reaction being

$$\underset{1}{CH_2OH}.\underset{2}{CHOH}.\underset{3}{CH_2OH} + 2NaIO_4 \xrightarrow{1,3} \underset{2}{HCHO} + HCOOH + 2NaIO_3 + H_2O.$$

The formic acid, derived from C atom 2 was oxidized with HgO and the CO_2 trapped in saturated $Ba(OH)_2$ (Topper & Hastings, 1949). The formaldehyde, derived from C atoms 1 and 3, was isolated, after the oxidation of the formic acid, as the Dimedone (5:5-dimethyl*cyclo*hexane-1:3-dione) derivative (Reeves, 1941). The degradation can be carried out conveniently on 50–100 mg. of glycerol. Glycerol labelled with known amounts of ^{14}C in either C atoms 1, 3, or in C atom 2, was not available to check the specificity of the method of degradation. It has been stated, however, by Topper & Hastings (1949) that, under the conditions described by them, formaldehyde is not oxidized by HgO.

RESULTS

The specific activity/time curves for both substances are shown in Fig. 1. It will be seen that they behave exactly as predicted on theoretical grounds by Zilversmit, Entenman & Fishler (1943) for the relationship between the specific activity/time curves of a labelled precursor (in this case lactose), and its immediate reaction product (in this case glycerol). The glycerol curve is always below the lactose curve until the former reaches its maximum, when it crosses the lactose curve and thereafter declines. The specific activities of both substances decline approximately exponentially from their maxima, the half-life for lactose being estimated graphically as 2·4 hr. and for glycerol as 3·0 hr. From these values the following 'turnover' times can be calculated: lactose, 3·5 hr. and glycerol, 4·3 hr. These values imply fairly rapid rates of synthesis of both lactose and glycerol.

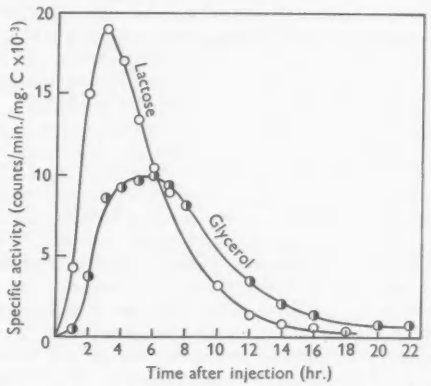

Fig. 1. Specific activities of lactose and glycerol isolated from the milk of a lactating goat which received an intravenous injection of 5 mc. of [*carboxy*-^{14}C]acetate.

A sample of the pooled glycerol was degraded to ascertain the position of the ^{14}C in the molecule. Table 1 shows that about 95% of the radioactivity

Table 1. *Distribution of* ^{14}C *in the carbon atoms of glycerol*

(For experimental details see text.)

Material examined	^{14}C content (counts/min./mg. carbon)
Carbon atoms 1 and 3	5750
Carbon atom 2	552
All carbon atoms as determined	4050
All carbon atoms as calculated from degradation	4117

of glycerol was accounted for by the isotope contents of carbon atoms 1 and 3, whereas carbon atom 2 contained only about 5% of the total ^{14}C content of the molecule.

DISCUSSION

Though the results are consistent with the theory that lactose is the immediate precursor of glycerol in the udder, we do not suggest that this is actually the case. It seems more likely that glucose is the immediate carbohydrate precursor of glycerol and that lactose is in very rapid equilibrium with glucose, and, moreover, is secreted very rapidly so that its specific activity at any moment is not measurably different from that of its precursor, glucose. This also follows from the observation of

French, Popják & Malpress (1952) that the specific activities of the glucose and galactose units in lactose are identical after the administration of [^{14}C]glucose or [^{14}C]starch to lactating rabbits.

The finding of radioactive lactose in the milk after the injection of $CH_3{}^{14}COONa$ into a lactating animal must be the result of either (or of both) of two processes: (*a*) incorporation of the isotope into blood glucose (in the liver) and transport to the udder; and (*b*) synthesis of hexose within the udder itself. Our experiment provided no information on the first of these processes. However, significant amounts of radioactivity are found in the blood glucose of lactating rabbits after the injection of either [^{14}C]acetate or of [^{14}C]pyruvate. On the other hand, the incorporation of acetate-carbon into lactose in the isolated udder has been shown to occur by a mechanism other than carbon dioxide fixation (Cowie *et al.* 1951) and, therefore, it seems probable that in our experiment on the intact animal the appearance of radioactive lactose was due, at least partly, to synthetic activities within the udder itself. The amount of incorporation of acetate-carbon into lactose and glycerol was fairly large as judged by the specific activity of both substances. The maximum radioactivities of the lactose and glycerol shown in Fig. 1 correspond to about $9{\cdot}5 \times 10^{-3}$ μc. ^{14}C/mg. carbon and 5×10^{-3} μc. ^{14}C/mg. carbon respectively. It is of interest that the highest radioactivities of the long-chain saturated and unsaturated fatty acids in the milk of this animal (observed 4 hr. after the injection of acetate) were 20×10^{-3} μc./mg. carbon and 6×10^{-3} μc./mg. carbon respectively, while the maximum radioactivity of the short-chain fatty acids was 70×10^{-3} μc./mg. carbon (cf. Popják *et al.* 1951, Fig. 3).

The metabolism of acetate by the citric acid cycle, which is known to operate in the mammary gland (Terner, 1951), provides a path for the entry of acetate-carbon into pyruvate, and hence, by the reversal of the glycolytic cycle, acetate-carbon can find its way into glucose (cf. Wood, 1948; Topper & Hastings, 1949). It appears that in our experiment too, ^{14}C from $CH_3{}^{14}COONa$ was incorporated into hexose by the same process. It is known that after the administration of $CH_3{}^{14}COONa$ the glucose units of liver glycogen contain practically all the isotope in carbon atoms 3 and 4 (Wood, 1948; Topper & Hastings, 1949). This is accounted for by the fact that the metabolism of [*carboxy*-^{14}C]acetate by the citric acid cycle yields pyruvate labelled in the carboxyl carbon which will enter into positions 3 and 4 of glucose. The breakdown of [3:4-^{14}C]-glucose into glycerol should yield [1:3-^{14}C]glycerol. The results of the degradation of glycerol shown in Table 1 are in complete harmony with this conception. The finding of some radioactivity in carbon atom 2 of glycerol indicates that positions 2 and 5 of the hexose must have also contained some ^{14}C.

In our experiment it is not certain whether the liver or the udder is the most likely site for the incorporation of acetate-carbon into glucose; the data shown in Fig. 1, however, make it seem very likely that the breakdown of glucose to glycerol occurs within the udder itself. Glycerol could be formed from glucose at the triosephosphate stage of the glycolytic breakdown. Dismutation of triosephosphate would yield glycerophosphate which could be dephosphorylated by the very active phosphomonoesterase present in mammary tissue (Folley & Kay, 1935). If lactose synthesis from small molecules occurs appreciably within the udder it might be argued that glycerol should appear as the precursor of lactose, since triosephosphate (presumably in equilibrium with glycerol) is an intermediate between pyruvate and hexose. The most likely explanation of the fact that lactose appears as the precursor of glycerol, and not the other way about as this argument would require, seems to be that the reaction triosephosphate → glycerol is much slower than triosephosphate → hexose, or else that the contribution to the milk lactose of radioactive glucose formed in the liver and carried to the udder in the blood must have greatly predominated over that of radioactive glucose formed in the gland itself. Further work is in progress to elucidate in detail the mechanisms of hexose formation in the mammary gland from various substrates.

While it may have been reasonably anticipated that glycerol in mammalian tissues is derived from glucose, just as in the fermentation of glucose by yeast, we do not know of any formal proof of this reaction in animals, other than that recorded by French & Popják (1951) and in this article.

SUMMARY

1. The specific activity/time curves for lactose and glyceride glycerol in the milk of a goat injected with [*carboxy*-^{14}C]acetate exhibit the mutual relationship characteristic of the curves for a labelled precursor (lactose curve) and its immediate reaction product (glycerol curve).

2. It is concluded that glucose (from which lactose is rapidly formed) is the carbohydrate precursor of glycerol in the udder.

3. The distribution of ^{14}C in the glycerol molecule (95 % in carbon atoms (1) and (3)) is consistent with the view that the acetate-carbon entered glucose by way of the citric acid cycle followed by the glycolytic process in reverse.

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The Hydrolysis of Succinyldicholine by Cholinesterase

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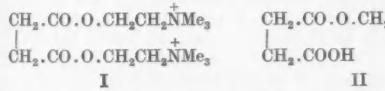
The dicholine ester of succinic acid (I) has recently found clinical application as a powerful curare-like drug with a short-lasting action and relative freedom from undesirable reactions (Bovet, Bovet-Nitti, Guarino, Longo & Marotta, 1949). Unlike natural curare, its action is potentiated, not antagonized, by eserine, and since it is also known to be hydrolysed *in vitro* by plasma cholinesterase (Glick, 1941; Bovet-Nitti, 1949), its transient action is probably due to destruction by cholinesterase *in vivo*. As the half ester (II) is without



I II

curare-like action, inactivation could conceivably be brought about either by simultaneous hydrolysis of both links of (I), giving a mixture of choline and succinic acid, or by hydrolysis to (II) as the intermediate or final product. The mechanism of the destruction of succinyldicholine by cholinesterase is thus of interest both from the pharmacological and enzymological points of view. We have studied the problem *in vitro* by means of chromatographic separation of the constituents of the reaction mixture at different stages of the hydrolysis. In earlier work (Whittaker, 1951) the solvent system used effected the separation of the two esters of succinic acid but not that of succinylmonocholine and choline, and these could only be distinguished by means of a reagent giving a coloration with carboxylic esters. A solvent mixture has now been found which gives good separation of all four of the possible constituents of the reaction mixture, namely succinyldicholine, succinylmonocholine, choline and succinic acid, and the chromatographic studies have been supplemented by a kinetic analysis, using the Warburg technique.

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METHODS

Source of enzyme. Horse serum cholinesterase was prepared by the method of Strelitz (1944) from commercial horse serum. The preparation corresponded to that obtained at stage 3 of her procedure and had initially a specific activity of 33 120 μl./ml. enzyme solution/hr., measured manometrically at pH 7·4 and 38° with acetylcholine (30 mM) as the substrate. The enzyme was stored at 0° with $CHCl_3$ as a preservative; its activity was unchanged by incubation without added substrate for several hours at 38°, but slowly declined during the course of the work to 23 600 units. Initial velocities are expressed as μmoles acid liberated/3 ml./hr. in presence of 23 600 units of enzyme activity.

Substrates. The choline esters and choline were employed as the perchlorates.

Paper chromatography. This was carried out as described in a previous paper (Whittaker & Wijesundera, 1952). Immersion in I_2 vapour (Brante, 1949; Marini-Bettolo-Marconi & Guarino, 1950) proved a useful general method, giving yellowish or brown spots with choline and its esters and succinic acid. The carboxylic ester reagent (Whittaker & Wijesundera, 1952) was used to detect the succinylcholine esters, and bromothymol blue, used as described by Brown (1950), detected succinylmonocholine and succinic acid as yellow spots. Whatman no. 4 filter paper was used throughout.

Table 1. *R_F values of choline and succinylcholines in various solvents*

(Compositions are by vol.; aliphatic alcohols n unless stated otherwise; upward irrigation in all cases.)

Solvent system	Succinyl-dicholine*	Succinyl-monocholine*	Choline*
Phenol-water	1·0	1·0	1·0
Acetone-acetic acid-water (8:1:1)	0·9	0·93	0·5
*cyclo*Hexanol-ethanol-water (4:2:1)	0·19†	0·41†	0·33†
Dioxan-water (9:1)	0·44†	0·53†	0·63†
Butanol-acetic acid-water (4:5:1)	0·24	0·71	0·71
Propanol-formic acid-water (8:1:1)	0·3	0·62	0·6
Propanol-formic acid-water (80:5:15)	0·46	0·49	0·43
*iso*Propanol-formic acid-water (8:1:1)	0·6	0·81	0·82
Butanol-propanol-water (4:2:1)	0·12	0·04	0·31
Propanol-water-conc. aqueous ammonia (sp.gr. 0·880) (8:1:1)	0·33	0·16	0·42
Propanol-benzyl alcohol-water (5:2:2)‡	0·30	0·17	0·45
Ethyl acetate-acetic acid-water (7:2:1)	0	0	0
Ethyl acetate-pyridine-water (7:2:1)	0	0	0
*cyclo*Hexanone-ethanol-water (4:2:1)	0	0	0

Collidine, dioxan-propanol-water (9:2:2), chloroform-acetic acid (8:2) sat. with water, acetone-water (5:1), benzy alcohol-ethanol-water (9:1:1), benzyl alcohol-water were all unsatisfactory.

* Perchlorates.

† Tailing.

‡ Higher water content in the propanol-benzyl alcohol-water mixture increased R_F values and reduced separation; increased benzyl alcohol content gave ill-defined spots.

Several solvent mixtures of the large number tried gave good separation between the two choline esters, but only one neutral mixture (*n*-propanol-benzyl alcohol-water, 5:2:2 by vol.) effectively separated all four possible components of the reaction mixture. Acid and alkaline solvents were avoided owing to the risk of hydrolysis on the paper. The results are summarized in Table 1.

Manometric technique. The hydrolysis of the succinylcholines was followed manometrically at 38° and pH 7·4 using an adaptation (Ammon, 1933) of the Warburg technique in which acid formed during hydrolysis liberates CO_2 from a $NaHCO_3/CO_2$ buffer. All solutions contained 0·2% (w/v) $NaHCO_3$, the total volume being 3 ml. The enzyme solution (usually 1 ml.) was contained in the side bulbs of Warburg flasks and the esters (neutralized where necessary) were in the main compartment. After attachment to the manometers, the flasks were gassed with 5% (v/v) CO_2 in N_2 (20 cm. water pressure for 2·5 min.). After equilibration in the thermostat for 20 min. the enzyme was tipped in and the first reading taken 3 min. later.

RESULTS

Hydrolysis of succinylcholines by cholinesterase

Fig. 1 shows the hydrolysis of succinylmonocholine and succinyldicholine by horse serum cholinesterase as a function of substrate concentration. Succinyldicholine shows some inhibition by excess substrate at higher concentrations. At the optimum, it is split at 3·4% of the maximum rate for acetylcholine. This is in good agreement with the figure of 4% as found by Glick (1941).

Succinylmonocholine is also hydrolysed by horse serum cholinesterase, though only very slowly (10% of the rate found with succinyldicholine). Its affinity for the enzyme is also less than that of the diester. As hydrolysis of the latter continues beyond 50% (Fig. 2), there are thus two possible mechanisms for the hydrolysis of the diester, both eventually leading to a mixture of choline and succinic acid. These are

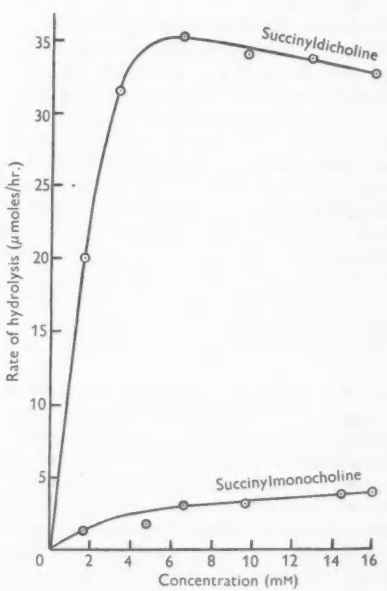

Fig. 1. Effect of substrate concentration on rates of hydrolysis of succinyldicholine and succinylmonocholine. Initial velocities measured over 5 min. and expressed as μmoles/hr.

(*a*) hydrolysis of both links simultaneously; (*b*) hydrolysis of one ester link with the formation of succinylmonocholine and choline followed by a slower hydrolysis of succinylmonocholine to choline and succinic acid.

Time course of hydrolysis of succinyldicholine

From Fig. 2, curve *A*, it will be seen that there is a marked change in the direction of the curve as 50% hydrolysis is reached, suggesting a two-stage reaction. The rate attained is equal to that obtained with an equimolar mixture of choline and succinylmonocholine (each 10 mM) (curve *B*); further, mixtures of succinyldicholine, succinylmonocholine and choline corresponding in composition to the reaction mixture at 25 and 40% hydrolysis on mechanism (*b*) give hydrolysis curves (Fig. 2, curves *C* and *D*) which

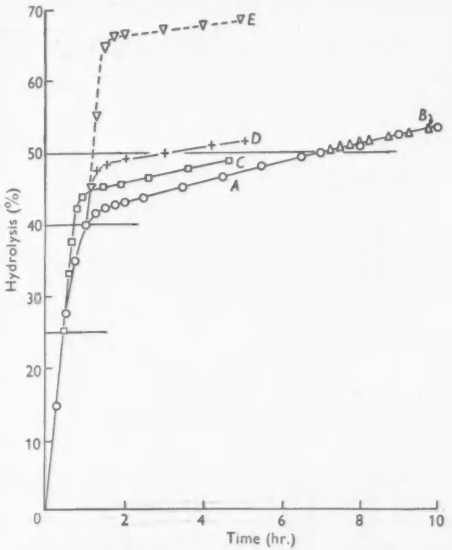

Fig. 2. The time course of hydrolysis of succinyldicholine (SCh_2) alone and mixed with succinylmonocholine (SCh), choline (Ch) and succinic acid (S). *A*, ○, 10 mM-SCh_2; *B*, △, 10 mM-SCh + 10 mM-Ch; *C*, □, 5 mM-SCh_2 + 5 mM-SCh + 5 mM-Ch; *D*, +, 2 mM-SCh_2 + 8 mM-SCh + 8 mM-Ch; *E*, ▽, 6 mM-SCh_2 + 8 mM-Ch + 4 mM-S.

follow the original curve fairly closely. By contrast, a mixture of succinyldicholine, choline and succinic acid corresponding to 40% hydrolysis on mechanism (*a*) is hydrolysed too fast to be consistent with the original hydrolysis curve (curve *E*). Curves *B*–*E* have been superimposed on curve *A* to show this more clearly. It seems likely, therefore, that the first stage in the hydrolysis of succinyldicholine is hydrolysis to succinylmonocholine and choline and that the rate which hydrolysis assumes at the 50% hydrolysis point is that of succinylmonocholine in the presence of choline.

Chromatographic analysis of the products of hydrolysis of succinyldicholine

For chromatographic analysis of the reaction mixture at different stages, five identical hydrolysis mixtures were set up. Hydrolysis was arrested in each at times corresponding respectively to 0, 10, 35, 50 and 60% hydrolysis of succinyldicholine by adding 0·1 ml. eserine solution to the Warburg flasks, giving a final concentration of 0·1 mM. Fig. 3 shows that this concentration of eserine stops the reaction in under 5 min. The reaction mixtures were adjusted to pH 7 by the cautious addition of 0·1 N-HCl, and 20 μl. of each solution (equivalent to approx. 100 μg. succinyldicholine) were pipetted on to the paper. Fig. 4 is a tracing of the resulting chromatograms. It will be seen by comparing chromatograms *a*–*e* that the succinyldicholine spot decreases in size and has disappeared at 50% hydrolysis while choline and succinylmonocholine, initially absent, are present in increasing amounts up to 50% hydrolysis. Thereafter, succinylmonocholine begins to break down. At 60% hydrolysis, the choline spot is larger than at 50% hydrolysis and succinic acid has now made its appearance on the chromatogram. Chromatogram *a* shows that protein and eserine do not interfere in the amounts present.

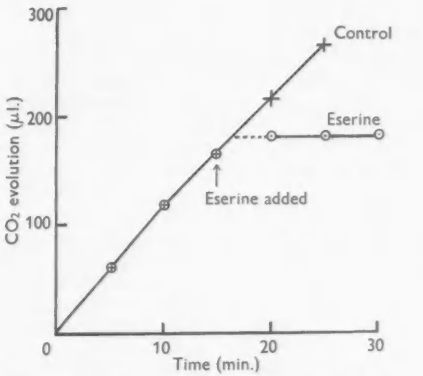

Fig. 3. The effect of 0·1 mM-eserine on the hydrolysis of 10 mM-succinyldicholine.

The slightly lower R_F values obtained with the constituents of the hydrolysates may be due to the presence of chloride ion introduced when the solutions were neutralized. It is known that chlorides have lower R_F values than the corresponding perchlorates (Whittaker & Wijesundera, 1952).

The enzymic breakdown of succinyldicholine in the presence of horse serum cholinesterase is thus clearly a two-stage process involving first the hydrolysis of one ester link with the formation of succinylmonocholine and choline and then the slower breakdown of succinylmonocholine to give another molecule of choline and succinic acid. Presumably the greater affinity of the dicholine ester for the enzyme assures its almost complete destruction before succinylmonocholine is attacked.

This is in marked contrast to the non-enzymic hydrolysis of succinyldicholine observed on omitting enzyme from the system used here. If this is allowed to occur to the extent of 20%, succinic acid as well as succinylmonocholine and choline are present in the hydrolysate (Fig. 4*g*).

THEORETICAL ANALYSIS OF THE HYDROLYSIS CURVE

An attempt has been made to give a theoretical treatment of the time course of hydrolysis of succinyldicholine by assuming (*a*) that the rate of hydrolysis of each ester is proportional to the concentration of the corresponding enzyme-substrate complex; (*b*) that the assumptions of the Michaelis theory hold good throughout the reaction and that, in consequence, the concentration of each enzyme-substrate complex is defined by the affinity of the various components of the reaction mixture for the enzyme; (*c*) that the succinate ion has a negligible affinity for the enzyme; (*d*) that inhibition by excess succinyldicholine (cf. Fig. 1) is negligible.

If e_0, e_1, e_2, e_3 denote the total concentration of enzyme, and the concentration of its complexes with succinyldicholine, succinylmonocholine and choline respectively, and if x_0, x, y, z, denote the initial concentration of succinyldicholine and the instantaneous concentrations of succinyldicholine, succinylmonocholine and choline respectively, the system is defined by the equations:

$$z = 2x_0 - 2x - y, \tag{1}$$

$$k_1 = (e_0 - e_1 - e_2 - e_3)\, x/e_1, \tag{2}$$

$$k_2 = (e_0 - e_1 - e_2 - e_3)\, y/e_2, \tag{3}$$

$$k_3 = (e_0 - e_1 - e_2 - e_3)\, z/e_3, \tag{4}$$

$$-\mathrm{d}x/\mathrm{d}t = ke_1, \tag{5}$$

$$-\mathrm{d}y/\mathrm{d}t = k'e_2 - ke_1, \tag{6}$$

where k's are constants.

Eliminating e_2 and e_3 from eqns. 2, 3 and 4, we obtain

$$e_1 = \frac{xe_0}{k_1(1 + x/k_1 + y/k_2 + z/k_3)},$$

and replacing z by the right-hand side of eqn. 1, we have

$$e_1 = \frac{xe_0/k_1}{1 + 2x_0/k_3 + x(1/k_1 - 2/k_3) + y(1/k_2 - 1/k_3)}. \tag{7}$$

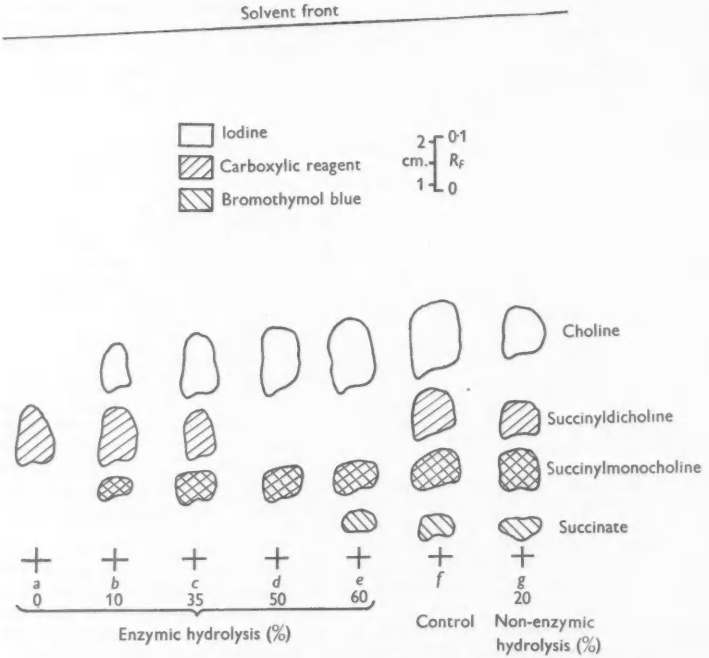

Fig. 4. Chromatogram of (*a*–*e*) succinyldicholine at various stages of enzymic hydrolysis; (*f*) a mixture of succinyldicholine and its hydrolysis products; (*g*) 100 μg. succinyldicholine after non-enzymic hydrolysis.

Similarly,

$$e_2 = \frac{ye_0/k_2}{1 + 2x_0/k_3 + x(1/k_1 - 2/k_3) + y(1/k_2 - 1/k_3)}. \tag{8}$$

By dividing eqn. 6 by eqn. 5, replacing e_1 and e_2 by the right-hand side of eqns. 7 and 8 and integrating, y may be obtained as a function of x, thus,

$$y = \frac{x_0^{1-k'k_1/kk_2}}{1 - k'k_1/kk_2}\, x^{k'k_1/kk_2} - \frac{x}{1 - k'k_1/kk_2}. \tag{9}$$

Eqns. 7 and 9 allow us to obtain e_1 as a function of x and using this value of e_1 in eqn. 5, we obtain a differential

equation which can be integrated without difficulty. Thus we find

$$t=\frac{k(k_1k_2-k_2k_3+k_1k_3)+k'k_1(k_3-2k_1)}{e_0kk_3(k'k_1-k_2k)}(x_0-x)$$
$$-\frac{kk_2(k_3-k_2)}{e_0k'k_3(k_1k'-k_2k)}\frac{x_0^{k_1k'/k_2k}-x^{k_1k'/k_2k}}{x_0^{(k_1k'/k_2k-1)}}$$
$$+\frac{k_1(k_3+2x_0)}{e_0kk_3}\ln(x_0/x).$$

From Fig. 1, $k_1=1{\cdot}5$ mM, $k_2=3{\cdot}5$ mM, $e_0k=36$ units, $e_0k'=4{\cdot}2$ units (maximum initial velocities of succinyldicholine and succinylmonocholine hydrolysis respectively);

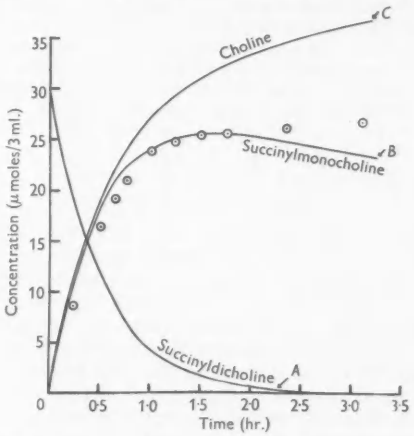

Fig. 5. Calculated concentrations of succinyldicholine, succinylmonocholine and choline as a function of time. ⊙, choline liberated in experimental hydrolysis of succinyldicholine.

also $k_3=3{\cdot}5$ mM, $x_0=30$ μmoles/3 ml. Using these values the third term only of the right-hand side of eqn. 10 need be considered; the function is expressed graphically in Fig. 5 (curve *A*). From eqns. 9 and 1, values of y and z corresponding to various values of x may be obtained; in Fig. 5 these are plotted against the values of t corresponding to these same values of x in order to obtain y and z as a function of t (curves *B* and *C*).

It will be seen that the curve of choline liberation, while not, as it should be, identical with the experimental hydrolysis curve, has the same general shape, with two sections corresponding to the hydrolysis of the dicholine and monocholine esters respectively and with a sharp change of direction between the two sections at a point where hydrolysis has proceeded to about 50%. The discrepancy between the theoretical and experimental curves may well be accounted for by inaccuracies in the values selected for the numerical coefficients and in the initial assumptions of the theory.

SUMMARY

1. The hydrolysis of succinyldicholine by horse serum cholinesterase has been studied kinetically and by paper chromatography of the hydrolysis products.

2. A solvent system has been found which will effect the separation on filter paper of all four of the constituents of succinyldicholine hydrolysates, i.e. the di- and monocholine esters of succinic acid, free succinic acid and choline.

3. Succinylmonocholine has been identified as a constituent of the reaction mixture. Succinic acid does not make its appearance in detectable amounts before 50% hydrolysis.

4. It is concluded that succinyldicholine is hydrolysed enzymically via succinylmonocholine. This is confirmed by the diphasic character of the hydrolysis curve and by kinetic studies.

5. The kinetics of such two-stage enzymic reactions is analysed mathematically.

We are grateful to Prof. D. Bovet for suggesting this problem and for hospitality to one of us (V.P.W.) at the Laboratorio di Chimica Terapeutica, Istituto Superiore di Sanità, Rome, where a preliminary study of the problem was made. We are also grateful to the Medical Research Council for a grant (V.P.W.) and to the University of Ceylon for a travelling fellowship (S.W.).

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Studies on the Metabolism of the Protozoa

2. THE GLYCOGEN OF THE CILIATE *TETRAHYMENA PYRIFORMIS* (*GLAUCOMA PIRIFORMIS*)

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(*Received* 21 *February* 1952)

A number of Protozoa synthesize intracellular carbohydrate, described as glycogen on account of the brown colour given with iodine; this polysaccharide may be diffusely distributed throughout the cell, or localized in 'glycogen vacuoles'. There do not seem to have been any investigations of the chemical nature of protozoal glycogen, nor of its physiological function. Recently, however, an opportunity for such an investigation arose from the observation, briefly reported in the preceding paper of this series (Ryley, 1951), that the protozoan *Glaucoma piriformis* may have an intracellular glycogen content as high as 22%. This protozoan will be referred to in the present study as *Tetrahymena pyriformis* in accordance with the new nomenclature advocated by Corliss (1952). The protozoal glycogen has been isolated in pure form and subjected to a detailed analysis.

MATERIALS AND METHODS

Ciliate preparations. Tetrahymena pyriformis was cultivated in a peptone medium as described in the following paper (Ryley, 1952). It was found that the peptone used in the preparation of the medium contained 0·44% of a KOH-resistant, ethanol-precipitable, glycogen-like material. Cells were harvested after 6 or 12 days growth, using a small angle centrifuge, or, in the case of larger preparations, the Sharples supercentrifuge.

Analytical methods. Total nitrogen was determined by digestion with H_2SO_4, using SeO_2 as catalyst. After digestion, ammonia was distilled *in vacuo* in the Parnas-Heller apparatus (Parnas & Heller, 1924), and estimated colorimetrically with Nessler's reagent. Phosphorus was estimated by the method of Fiske & Subbarow (1925), after digestion of a sample with 60% $HClO_4$ and a little HNO_3. Glycogen was estimated by the method of Good, Kramer & Somogyi (1933). Reducing sugar was estimated iodometrically by the method of Somogyi (1945), while glucose was determined as the loss in reducing value of a sample after treatment with glucose oxidase and catalase (Mann, 1946). The glycogen end-group assay was carried out by the method of KIO_4 oxidation developed by Halsall, Hirst & Jones (1947), as modified by Bell & Manners (1952). β-Amylolysis of glycogen was effected by treating the glycogen with crystalline sweet potato β-amylase, kindly supplied by Prof. C. S. Hanes, F.R.S., as described by Bell & Manners (1952). Molecular weight determinations were made by the light-scattering method, as described by Harrap & Manners (1952). We are indebted to Dr B. S. Harrap for the measurement of the turbidity of the glycogen.

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RESULTS

Glycogen content of the cells

After growth in a peptone medium of low carbohydrate content, the cells stained a uniform brown colour with iodine. Growth was accompanied by the production of free ammonia; after 6 days' growth, the ammonia content of the medium, as determined by steam distillation *in vacuo*, had increased from 42 to 88 μg./ml., there being a corresponding slight increase in the pH. Cells were harvested after 6 and after 12 days' growth at 30°, washed in 0·45% saline and then water, and made to a thick suspension in water; glycogen was estimated in duplicate 1 ml. samples of this suspension. Such analyses gave cell glycogen contents of 16, 14 and 17% for three 6-day cultures, and 23 and 22% for two 12-day cultures (on a dry weight basis).

Isolation and purification of glycogen

Five large-scale glycogen preparations were carried out, using in all a total of 130 l. of 12-day culture. The cells were harvested, treated with 2 vol. 30% (w/v) KOH at 100° for 30 min., cooled, centrifuged to remove a little insoluble material, and the crude glycogen precipitated with 1·1 vol. absolute ethanol. The precipitate was removed on the centrifuge, washed with 60%, 80% and absolute ethanol, and ether, and dried *in vacuo* over P_2O_5. In this way, a total of 4·8 g. crude glycogen were obtained. This product contained 0·52% N and 0·32% P, and was 66% pure on a basis of glucose liberated by acid hydrolysis. Further purification was achieved by precipitation with 80% (w/v) acetic acid (Bell & Young, 1934). Crude glycogen (4·8 g.) was dissolved in 130 ml. water; a brown, gelatinous, insoluble residue was removed on the centrifuge. Glacial acetic acid (520 ml.) was added to the supernatant, and the precipitate centrifuged off. It was dissolved in 61 ml. water, a little insoluble material was removed, and the glycogen precipitated by adding 244 ml. glacial acetic acid. This precipitation with acetic acid was repeated twice more, and the purified glycogen washed twice in 200 ml. of 80%, 90%

Table 1. *A comparison of the properties of* Tetrahymena, *rabbit-liver and rabbit-muscle glycogens*

(Data for rabbit-liver and rabbit-muscle glycogens taken from Bell (1948), Bell & Manners (1952) and Harrap & Manners (1952).)

Property	Source of glycogen		
	T. pyriformis	Rabbit liver	Rabbit muscle
$[\alpha]_D$ (water)	+195°	+198°	+196°
Iodine coloration	Yellow-brown	Red-brown	Purple-brown
Unit chain length:			
(*a*) KIO_4 method	13	13	13
(*b*) Methylation method	—	12	13
Conversion to maltose by β-amylase (%)	44	43	45
Molecular weight (light scattering)	$9{\cdot}8 \times 10^6$	$6{\cdot}8 \times 10^6$	$2{\cdot}8 \times 10^6$

and absolute ethanol and in ether. After a preliminary drying at 37°, the glycogen was dried *in vacuo* over P_2O_5 for 3 hr. at 100°. This gave 3·12 g. of purified glycogen.

Characterization of purified glycogen

The purified glycogen was a white powder, freely soluble in water, forming an opalescent solution, which gave a yellow-brown colour with dilute iodine. The iodine-staining power of the glycogen was quickly destroyed on treatment with salivary α-amylase, this being accompanied by the liberation of reducing sugar. The purified, dry glycogen had $[\alpha]_D^{18°}$ +195° in water (2 dm. tube, *c*, 0·3); N, 0·095; P, 0·069; ash 0·46 %; reducing value 0·22 % that of an equivalent weight of maltose. In Table 1, the properties of this purified glycogen are summarized, and compared with those of rabbit-liver and rabbit-muscle glycogens.

Glucose content. Hydrolysis of the glycogen for 2 hr. in 0·6 N-hydrochloric acid, followed by determination of the reducing value, indicated that the purified glycogen was 97 % pure, assuming it to be a polymer of glucose. That glucose was the only sugar liberated by acid hydrolysis was demonstrated by incubating samples of the hydrolysate with phosphate buffer (pH 5·6), and a well washed suspension of baker's yeast, or a mixture of glucose oxidase and catalase. Incubations were carried out in small conical flasks, which were shaken for 3 hr. at 37°; the yeast cells were then centrifuged off. The resulting solutions were non-reducing, indicating that all the sugar initially present was fermentable by yeast, and oxidizable by glucose oxidase.

Acid hydrolysis. The behaviour of *Tetrahymena* glycogen on acid hydrolysis was compared with that of rabbit-liver glycogen and starch. Both glycogens were completely hydrolysed by 0·6 N-hydrochloric acid at 100° in 60 min., while during the same time starch was 92 % hydrolysed. In 0·1 N-hydrochloric acid, both glycogens were hydrolysed at an identical rate (50 % in 150 min.) while starch was hydrolysed somewhat faster (50 % in 90 min.) (Fig. 1).

Unit chain length. On complete oxidation with potassium periodate 211·3 mg. glycogen gave 4·6 mg. formic acid. This corresponds to a unit chain length of 13 glucose residues.

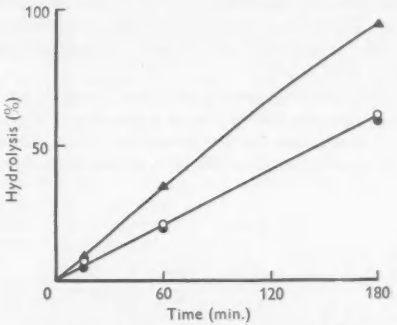

Fig. 1. Hydrolysis of starch, rabbit-liver glycogen and *Tetrahymena* glycogen in 0·1 N-HCl at 100°; ▲—▲, starch; ●—●, *Tetrahymena* glycogen; ○—○, rabbit-liver glycogen.

β-Amylolysis. Glycogen from *Tetrahymena* (43·6 mg.), on treatment with crystalline β-amylase, was converted to maltose as follows: 38 % in 30 min., 42 % in 135 min., 44 % in 20 hr., and 44 % in 47 hr.

Molecular weight. Light-scattering determinations indicated a molecular weight of $9{\cdot}8 \times 10^6$.

DISCUSSION

Most free-living Protozoa synthesize some type of intracellular carbohydrate, and it is interesting to compare the different ways in which these carbohydrate reserves arise. In a great number of cases, intracellular carbohydrate takes the form of starch granules, the production of which is associated with the presence of plastids in the cell. This starch may have a photosynthetic origin, or may be elaborated from simple carbon compounds present in the

growth medium, usually short-chain fatty acids. Polysaccharide formation in the flagellate *Polytomella cœca* has been studied in some detail. Rabinovich (1938) observed that starch formation by this flagellate, grown in a synthetic medium containing asparagine as nitrogen source, and acetate as carbon source, was associated with a sub-cuticular, reticulate leucoplast. Lwoff, Ionesco & Gutmann (1950) found that the organism, although it could not utilize free sugars, contained a phosphorylase system which would convert glucose-1-phosphate to polysaccharide. They prepared a quantity of this protozoal polysaccharide, which was examined chemically by Bourne, Stacey & Wilkinson (1950). It was found that the polysaccharide, which stained purple with iodine, was a starch-like substance, having an amylopectin content of about 85%. Oxford (1951) observed that a number of large holotrichous ciliates, found in the sheep rumen, were able to ferment simple sugars present in the food of the host, with the formation of intracellular carbohydrate granules; this carbohydrate was composed entirely of glucose, and stained a brownish purple colour with iodine.

With *Tetrahymena pyriformis*, growth of the ciliate and the formation of intracellular glycogen are accompanied by the liberation of free ammonia in the medium. In view of the low carbohydrate content of the growth medium, it seems possible that intracellular carbohydrate may arise from the products of protein hydrolysis and oxidation. This glycogen forms an energy store, utilizable under conditions of unfavourable oxygen tension (Ryley, 1952). From the table, it can be seen that the glycogen synthesized by *T. pyriformis* is very similar to mammalian glycogens, except that it has a rather higher molecular weight.

SUMMARY

1. A glucose-containing polysaccharide has been isolated from the ciliate *Tetrahymena pyriformis* (*Glaucoma piriformis*), grown in a peptone medium without added carbohydrate.

2. The purified polysaccharide shows all the properties of a typical animal glycogen. It has a molecular weight of $9{\cdot}8 \times 10^6$, $[\alpha]_D^{18°} + 195°$, and a unit chain length of 13 glucose residues.

3. Hydrolysis of the glycogen by crystalline sweet potato β-amylase gives 44% conversion to maltose.

We are indebted to Dr D. J. Bell and Dr T. Mann, F.R.S., for much helpful advice and discussion during the course of this work. We gratefully acknowledge the award of Research Studentships from the Agricultural Research Council (to D.J.M.), and from the Medical Research Council (to J.F.R.).

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Studies on the Metabolism of the Protozoa

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During the past 20 years, numerous studies have been carried out on the nutritional requirements and metabolic activities of certain small, holotrichous ciliates, known by a variety of names, which can be grown in bacteria-free pure cultures. As a result of morphological studies carried out with twenty-nine different cultures of these ciliates, Corliss (1952) has concluded that they are nearly all different strains of one and the same organism, which should in his opinion be called *Tetrahymena pyriformis*. In the present paper, the '*Glaucoma piriformis*' of A. Lwoff (1932), M. Lwoff (1934) and Lawrie (1935) and the '*Tetrahymena geleii*' of Chaix, Chauvet & Fromageot (1947) will be referred to as *Tetrahymena pyriformis* (GL), the '*Colipidium campylum*' of Wilber & Seaman (1948) and Seaman (1949), and the '*Tetrahymena geleii*' of Seaman (1950, 1951) will be referred to as *Tetrahymena pyriformis* (S), the '*Colipidium campylum*' of Hall (1941) will be referred to as *Tetrahymena pyriformis* (H), the '*Tetrahymena geleii*' of Pace & Ireland (1945) and Pace & Lyman (1947) will be referred to as *Tetrahymena pyriformis* (W), while the '*Tetrahymena geleii*' of Baker & Baumberger (1941) and Niel, Thomas, Ruben & Kamen (1942) will be referred to as *Tetrahymena pyriformis* (T); Kidder & Dewey (1945) give the origins of some of these strains.

A striking chemical characteristic of *T. pyriformis* (GL) is the unusually high content of intracellular glycogen (Ryley, 1951; Manners & Ryley, 1952). In the present study, the anaerobic metabolism of *T. pyriformis* is shown to depend on a phosphorylative breakdown of this reserve of glycogen, with succinic, lactic and acetic acids as end products, while the aerobic metabolism is shown to depend on processes other than glycogen breakdown.

MATERIAL AND METHODS

Organism. The culture of *Tetrahymena pyriformis* (GL) was kindly supplied by Dr E. G. Pringsheim of the Botany School, Cambridge; it was the strain described by Lwoff (1932) under the name *Glaucoma piriformis*. The organism was cultivated in a sterile medium (pH 7·3) containing 1% (w/v) 'Oxoid' brand bacteriological peptone and 0·4% (w/v) NaCl. Cultures were maintained in tubes at room temperature, sub-inoculations being made every 2 weeks. For experimental studies, cultures were grown at 30°, in flasks containing 2–4 l. medium, with an air space of 1 l. The organisms were harvested after 6 days' growth, using a small angle centrifuge; it was found necessary to use a centrifuge which came to rest quickly, and to remove the supernatant immediately by suction. With most centrifuges tried, although the ciliates were sedimented readily, by the time the centrifuge had stopped the organisms had swum back into the supernatant. The centrifuged cells were washed and suspended in either 0·45% (w/v) NaCl, a Ringer-phosphate solution containing 0·047 M-NaCl, 0·002 M-KCl, 0·001 M-$MgSO_4$ and 0·012 M-phosphate buffer (pH 7·3), or a Ringer-bicarbonate solution containing 0·05 M-NaCl, 0·003 M-KCl, 0·001 M-$MgSO_4$ and 0·016 M-$NaHCO_3$, which when used in conjunction with a gas mixture of 5% CO_2–95% N_2 (v/v), had a pH of 7·3. Homogenates were made by washing the organisms in water, and then shaking a thick aqueous suspension with glass beads in the Mickle disintegrator for 5 min. Cell-free preparations were obtained by centrifuging this homogenate for 15 min. at 11 000 rev./min. in the Servall angle centrifuge. The supernatant was a clear, light-brown fluid, free from microscopically visible particles.

Materials. Glucose oxidase and catalase were kindly supplied by Dr E. F. Hartree. The glycogen used was a purified specimen prepared from *Tetrahymena pyriformis* (GL), described by Manners & Ryley (1952). Adenosinetriphosphate (ATP) was prepared from the barium salt, kindly supplied by Dr E. C. Slater. Potassium glucose-1-phosphate and calcium fructose-1:6-diphosphate were gifts from Dr T. Mann.

Spectroscopic observations. These were made by means of a microscope fitted with a Zeiss microspectroscope ocular.

Purpurogallin number (P.Z.). This was determined by the method of Sumner & Gjessing (1943), slightly modified. A mixture of 2 ml. 5% (w/v) pyrogallol solution, 2 ml. 0·5 M-phosphate buffer (pH 6·0), 1 ml. 1% (v/v) H_2O_2 and 15 ml. water were warmed to 20° in a beaker. An aqueous suspension (1 ml.) of the ciliate, which had been twice frozen and thawed to disrupt the cells, was added, and the mixture incubated at 20° for 5 min.; the reaction was stopped by the addition of 1 ml. 2 N-H_2SO_4. As much protein was present, and interfered with the extraction of purpurogallin, 1 ml. 100% (w/v) trichloroacetic acid was added, and the mixture centrifuged. The supernatant was extracted twice with ether, and the extract diluted to 25 ml. with ether. Purpurogallin was estimated photoelectrically in the

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extract. The P.Z. is defined as mg. purpurogallin formed per mg. material under the standard conditions used.

Metabolic experiments. These were carried out in Warburg manometers, using cups (vol. 20–25 ml.) fitted with two side arms. Gas phases of air or 5% CO_2–95% N_2 (v/v) were used; the experiments were mostly carried out at 37°. The effect of CO on respiration was studied in Barcroft differential manometers at 19° and at 30°. Metabolic rates were based on the N content of the suspensions, being expressed as q_{O_2} and $q^{N_2}_{CO_2}$, i.e. as μl. gas/mg. N/hr. It was found that 1 mg. ciliate N was equivalent to 8·9 mg. dry weight, or about 80 mg. wet weight of cells.

Estimation of metabolites. Glycogen was estimated by the method of Good, Kramer & Somogyi (1933). Reducing sugar was estimated iodometrically by the method of Somogyi (1945), in $Ba(OH)_2$-$ZnSO_4$ filtrates; glucose was determined from the loss in reducing value of such a filtrate after treatment with glucose oxidase and catalase (Mann, 1946), while fructose was estimated colorimetrically (Roe, 1934). Fructose estimations were also carried out in trichloroacetic acid filtrates to determine the total of free and phosphorylated sugar; values for phosphofructose were calculated from the difference in fructose content of these two filtrates. Phosphate fractions were estimated by the method of Fiske & Subbarow (1925); total phosphorus was estimated after digestion with 60% $HClO_4$ and a little HNO_3. Using trichloroacetic acid filtrates, pyruvic acid was estimated by the colorimetric method of Friedemann & Haugen (1943), and lactic acid by the method of Barker & Summerson (1941). Acetic acid was estimated by distillation of a tungstic acid filtrate (Buchanan, Sakami, Gurin & Wilson, 1947), while ethanol was determined by the bichromate method of Nicloux, le Breton & Doutcheff (1934). Succinic acid was estimated manometrically by means of a washed horse-heart preparation of succinic oxidase.

RESULTS

Aerobic metabolism

The free-living protozoan *T. pyriformis* (GL), although it occurs naturally at a much lower temperature, is able to maintain constant motility and metabolism at 37° for a period of several hours at least. It was thought preferable to work at this higher temperature, rather than 22° or similar temperatures utilized by previous workers, as this reduced the duration of the experiments. The organisms were cultivated at 30°, which was found to be the optimum temperature for growth; at 37°, although the ciliates flourished, growth was not quite as good as at 30°.

Respiratory activity. In a non-nutrient medium at 37°, the ciliate respired with a q_{O_2} of 480 (mean of seven values, ranging from 380 to 565). From Fig. 1 it can be seen that the respiration continued at an almost linear rate for 60 min.; other experiments have shown no marked decrease in respiration after 5 hr. in a similar system. Fig. 1 also shows that respiration proceeds at an almost linear, but reduced rate (q_{O_2} = 225) at 25°. Although the ciliate maintains a high endogenous respiration in the absence of extracellular nutrients, several added compounds were able to increase the O_2 uptake still further. Thus, as shown in Table 1, acetate, propionate, butyrate, and to a lesser extent pyruvate, increased the respiration, while glucose, ethanol, and a number of acids associated with the tricarboxylic acid

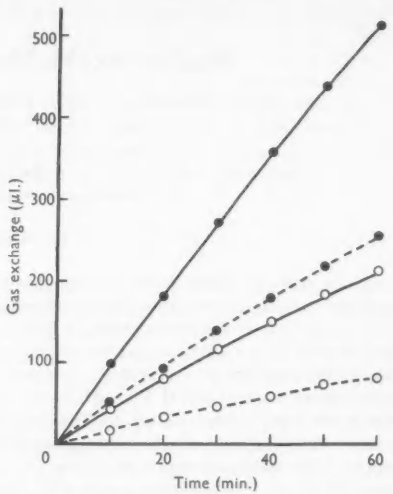

Fig. 1. Aerobic and anaerobic metabolism of *T. pyriformis* (GL). Each manometer cup contained 1·14 mg. ciliate N in a volume of 1·5 ml.; gas phase air or 5% CO_2–95% N_2; ●—●, O_2 uptake (μl.) at 37°; ●- - - -●, O_2 uptake (μl.) at 25°; ○—○, anaerobic CO_2 production (μl.) at 37° in bicarbonate buffer; ○- - - -○, anaerobic CO_2 production (μl.) at 25°.

Table 1. *Effect of extracellular substrates on respiration*

(Respiration of ciliates in Ringer-phosphate solution (pH 7·3) followed for 30 min.; 0·01 M substrate then added from side bulb, and respiration followed for a further 60 min. Table gives O_2 uptakes observed during this latter period. Total vol. 1·5 ml.; temp. 37°; gas phase air.)

Substrate added	O_2 uptake (μl./hr.)	Stimulation (%)
Exp. 1 (0·5 mg. ciliate N)		
None	206	0
Glucose	226	10
Ethanol	213	3
Sodium citrate	223	8
Sodium succinate	219	6
Sodium formate	218	6
Sodium acetate	385	87
Sodium propionate	400	94
Sodium butyrate	406	97
Exp. 2 (0·57 mg. ciliate N)		
None	265	0
Sodium succinate	300	13
Sodium malate	309	16
Sodium fumarate	275	4
Sodium malonate	276	4
Lithium lactate	308	16
Sodium pyruvate	368	39
Pyruvate + malonate	385	45
Pyruvate + malonate + fumarate	385	45
Sodium acetate	454	71

cycle had little effect. The dicarboxylic acids had even less effect on the respiration when tested at pH 6·0, or if added to organisms which had been starved prior to use by keeping in Ringer-phosphate solution for 20 hr. at room temperature. With one particular ciliate preparation, which during a period of 60 min. consumed 23 μmoles O_2, it was found that 0·01 M-glucose increased the O_2 uptake by 1·96 μmoles or 8%, while 3·87 μmoles glucose had disappeared. The oxidation of 3·87 μmoles glucose would require a much greater quantity of O_2 than the extra uptake of 1·96 μmoles observed. This may indicate a sparing of the unknown substrate responsible for endogenous respiration, or may indicate an assimilation of the sugar.

18·5 μmoles glucose; had the oxidation been incomplete, the glycogen utilization would have been even greater.

Effect of inhibitors on respiration and motility. The sensitivity of endogenous respiration to a number of inhibitors was studied; the results are summarized in Table 2. The presence of cyanide ($0{\cdot}46 \times 10^{-3}$ M), using the balanced KOH-KCN mixtures in the centre well as recommended by Robbie (1946), reduced respiration by only 18%; this inhibition was maintained over a period of 5 hr. On another occasion, in the presence of glucose, the same concentration of cyanide gave a 34% inhibition of O_2 uptake. CO ($CO/O_2 = 19$), had no inhibitory effect on respiration in the dark, at either 19 or 30°, but rather gave rise to a small

Table 2. *Effect of inhibitors on respiration*

(Each manometer cup contained about 0·6 mg. ciliate N in a total vol. of 1·5 ml.; gas phase air; temp. 37°. Inhibitor added from side bulb, and O_2 uptake over a period of 1 hr. compared with that of control.)

Inhibitor	Concn. (M)	Respiration (as % that of control) pH 7·3	pH 6·0	pH 5·5
None	—	100	100	100
Potassium cyanide	0·00046	82	—	—
Carbon monoxide (30°)	($CO/O_2 = 19$)	105	—	—
Sodium azide	0·01	—	17	40
Sodium azide	0·001	—	143	272
Sodium azide	0·0001	—	119	157
2:4-Dinitrophenol	0·001	—	28	—
2:4-Dinitrophenol	0·0002	—	65	—
2:4-Dinitrophenol	0·0001	—	144	—
2:4-Dinitrophenol	0·00002	—	143	—
Hydroxylamine	0·002	70	—	—
Sodium iodoacetate	0·0001	69	—	—
Sodium arsenite	0·01	38	—	—
Sodium fluoride	0·02	100	—	—
Phlorrhizin	0·01	88	—	—
2:2′-Dipyridyl	0·0003	96	—	—
4:4′-Dipyridyl	0·0003	95	—	—
8-Hydroxyquinoline	0·0003	83	—	—
Phenylthiourea	0·0003	100	—	—

Nature of endogenous respiration. A determination of the respiratory quotient was made, using the direct method of Warburg on organisms in a Ringer-phosphate medium; corrections were applied both for the initial and the final bound CO_2 in the medium. This gave an R.Q. value of 0·85, which suggests that endogenous respiration is probably not dependent on carbohydrate metabolism. A similar conclusion can be derived from experiments in which intracellular glycogen was estimated before and after a period of active respiration. Six manometers were set up, each containing 1 mg. cellular N in Ringer-phosphate solution. After manometric equilibration, 2 N-H_2SO_4 was tipped from the side bulb into three of the flasks, while the ciliates in the other three flasks were allowed to respire for a period of 150 min. Respiration was stopped by the addition of H_2SO_4, and glycogen was estimated in the contents of each of the flasks. The initial glycogen present was equivalent to 45·2 μmoles glucose. During the incubation, a total of 111 μmoles O_2 was consumed by the ciliates in the three flasks, while the decrease in glycogen content amounted to only 1·79 μmoles glucose. Had the O_2 uptake observed been due to the complete oxidation of carbohydrate, there would have been a glycogen disappearance corresponding to

stimulation. Azide, another potent inhibitor of the cytochrome oxidase system, was used at pH 6·0 and 5·5, but acted as an inhibitor only at high concentrations. Indeed, at concentrations of 0·001 M and less, azide caused a pronounced stimulation of the O_2 uptake. Motility studies, however, showed that azide inhibited motility to a small extent at a concentration of 2×10^{-5} M, and that higher concentrations had a larger effect; the influence of azide on motility increased with the time of incubation. 2:4-Dinitrophenol had similar effects to azide on respiration; concentrations greater than 0·0002 M inhibited respiration at pH 6·0, while concentrations less than 0·0001 M stimulated respiration. In all cases, there was an adverse effect on motility; in the presence of 0·001 M-dinitrophenol, motility was abolished within 30 min. at room temperature, while in 0·0002 M-dinitrophenol, motility was gradually reduced, until the organisms were completely immotile after 230 min. The controls to these experiments showed that respiration at pH 6·0 was as good as that observed at pH 7·3, but that at pH 5·5, O_2 uptake was reduced by 30%.

Respiration and motility were insensitive to high concentrations of fluoride, while arsenite had little effect at a concentration less than 0·01 M; this concentration of

arsenite was sufficient to render the ciliates almost stationary after 100 min. at room temperature, while a concentration of 0·003 M had a smaller, but noticeable, effect after this time. Iodoacetate at a concentration of 3×10^{-5} M had no effect on respiration and motility, but 0·0001 M-iodoacetate reduced both considerably over a period of 60 min.; 0·0003 M-iodoacetate began to produce inhibitory effects after 15 min. incubation, and within 1 hr. the organisms were completely immotile. Phlorrhizin and the metal-binding substances tested were without noteworthy effect, save that 8-hydroxyquinoline caused a slight reduction in motility. In all cases, loss of motility was an irreversible process, being accompanied by damage to the cells.

Haem compounds

Cytochrome system. Direct spectroscopic examination of a suspension of *T. pyriformis* (GL) shows the presence of a number of haem pigments. Under conditions of good aeration, the most prominent feature of the spectrum is the α-band of oxyhaemoglobin (Keilin & Ryley, 1951). When reduced with a small amount of sodium dithionite ($Na_2S_2O_4$), a cell suspension examined at room temperature showed: (*a*) A narrow, weak cytochrome *c* band at 550 mμ. (*b*) A strong cytochrome *c* band at 551–553 mμ. (*c*) A weak cytochrome *b* band at 563 mμ. (*d*) An exceedingly weak band at 605 mμ., probably due to cytochrome *a*. (*e*) Double β-bands at 521 and 530 mμ. (*f*) A very weak and diffuse band at 580–590 mμ., possibly due to cytochrome a_1, or to incompletely reduced haemoglobin. This band moved to 568–578 mμ., as a weak and very diffuse band, in the presence of CO; the other bands were not affected by CO. (*g*) A very faint band at 635 mμ., probably due to methaemoglobin, derived by oxidation of some of the haemoglobin.

Manometric experiments showed that a homogenate of the ciliate was unable to catalyse the oxidation of *p*-phenylenediamine, either in the presence or in the absence of added cytochrome *c*. In connexion with this lack of cytochrome oxidase activity, the results of Table 2 concerning the effects of azide, cyanide and CO on respiration, should be noted.

Peroxidase. Lawrie (1935) reported that *T. pyriformis* (GL) contained a peroxidase. The peroxidase activity of the protozoan has been confirmed, and the purpurogallin number determined. Saturated ethanolic benzidine (1 ml.) was mixed with 1 ml. water, 6 drops glacial acetic acid, and 1 ml. of a suspension of the ciliate. A few drops of H_2O_2 were added; the mixture rapidly became blue in colour. Microscopic examination immediately after adding H_2O_2 showed that the cells became uniformly blue throughout, but that this colour soon diffused out of the cell. (Similar experiments with *Paramecium caudatum* showed a concentration of the blue colour in and around unidentified granules in the cell.) Boiled organisms gave only a slight production of colour, indicating that the benzidine reaction was mainly due to a thermolabile enzyme, rather than to the intracellular haematin as such. Similar colour-producing reactions were obtained with other phenolic compounds, such as pyrogallol, *p*-phenylenediamine, guaiacum or pyrocatechol, when tested with H_2O_2 in 0·1 M-phosphate buffer (pH 7·3). In each case, colour production was dependent on the addition to the system of H_2O_2 and cell suspension. During these experiments, a considerable production of gas was observed, due to decomposition of the peroxide by catalase. Using the modified method of Sumner & Gjessing (1943), it was found that 14·4 mg. dry wt. of cells formed 0·475 mg. purpurogallin, giving a P.Z. of 0·033.

Cell homogenates were able to oxidize reduced cytochrome *c* peroxidatically. A small amount of dilute cytochrome *c* solution was reduced by adding a trace of *p*-phenylenediamine. Cell homogenate was then added, and the mixture examined spectroscopically. The intense band of cytochrome *c* at 550 mμ. did not fade in spite of aeration for at least 1 hr., indicating that no active cytochrome oxidase was present. When H_2O_2 was added to the system, the cytochrome *c* band quickly disappeared, reappearing when all the peroxide had been utilized; further addition of H_2O_2 caused the band to disappear once more.

Anaerobic metabolism

Under anaerobic conditions, *Tetrahymena pyriformis* (GL) is able to survive and maintain motility for prolonged periods of time. A suspension of the ciliate in Ringer-bicarbonate solution was placed in a Thunberg tube, which was evacuated and filled four times with an O_2-free CO_2/N_2 mixture. Normal motility was maintained by a large proportion of the cells (examined microscopically with a $\frac{2}{3}$ in. objective while in the tube) for at least 72 hr., although a number of the organisms died during this time; a similar picture was obtained with a control suspension exposed to the air. Initially, all the cells stained a dark-brown colour with dilute iodine, but many of those which had been kept under anaerobic conditions for 72 hr. gave very little coloration with iodine; the cells kept for the same period of time under aerobic conditions still gave a good iodine stain. When suspended in a Ringer-bicarbonate medium under anaerobic conditions, the ciliate produced acid (Fig. 1). From six experiments carried out at 37°, a mean $q^{N_2}_{CO_2}$ of 180 was observed (range 123–238), while at 25°, a $q^{N_2}_{CO_2}$ value of 70 was obtained. This liberation of CO_2 was increased by 33% in the presence of 0·01 M-glucose, 35% of the added glucose being utilized.

Nature of anaerobic endogenous metabolism. Under anaerobic conditions the ciliate fermented its intracellular glycogen reserves and maintained a high degree of motility. Six manometers were set up, each with 1 mg. ciliate N in 1·5 ml. Ringer-bicarbonate solution, and a CO_2-N_2 gas phase. After equilibration, acid was tipped into three of the flasks in order to estimate the initial bicarbonate and after 110 min. acid was tipped into the other flasks to estimate residual bicarbonate. Estimations showed that the glycogen reserves had been depleted by 32% during the fermentation, while 55·0 μequiv. acid had been produced, and 7·05 μmoles CO_2 had been assimilated, i.e. for each glucose unit of glycogen metabolized, 2·75 equiv. of acid were formed, with the assimilation of 0·35 molecules of CO_2.

A similar experiment was carried out on a somewhat larger scale, the flask contents being analysed for glycogen, succinic, lactic, pyruvic and acetic acids and ethanol. From Table 3 it can be seen that the glycogen was mainly converted to succinic acid, with smaller amounts of lactic and acetic acid. The metabolites estimated accounted for 91% of the carbon supplied, and for 97·5% of the CO_2 liberated by acid formation.

Effect of inhibitors on anaerobic metabolism. From Table 4 it can be seen that of the seven compounds tested as possible inhibitors of the fermentation, only iodoacetate had a marked effect. In the presence of 0·001 M-iodoacetate, CO_2 evolution was reduced by 75% over the period of 60 min.

studied; the metabolism stopped within 20 min. of adding the inhibitor. In contrast to the anaerobic metabolism of glucose by *Trypanosoma lewisi* (Ryley, 1951), which is highly sensitive to the metal-binding substances 2:2′-dipyridyl and 8-hydroxyquinoline, these same compounds were found to stimulate the rate of an aerobic acid production by *Tetrahymena pyriformis* (GL).

Table 3. *Anaerobic glycogen fermentation by* Tetrahymena pyriformis *(GL)*

(Ciliate N 8·8 mg. was incubated for 120 min. in a total vol. of 18 ml. Ringer-bicarbonate solution, using six manometers; gas phase 5% CO_2–95% N_2; temp. 37°. Reaction stopped, and residual bicarbonate estimated by adding 0·4 ml. 2N-H_2SO_4 to each flask. Contents of flasks neutralized, pooled, and deproteinized for estimation of metabolites. Yields expressed in terms of moles metabolite found per glucose unit of glycogen disappearing, from which values for carbon and acid recoveries have been calculated. During the fermentation, a total of 71·1 μmoles glucose (as glycogen) were used, and 28·9 μmoles CO_2 were fixed.)

Metabolite	Moles	Carbon (g. atoms)	Acid (g. equiv.)
Succinic acid	0·94	3·76	1·88
Lactic acid	0·47	1·41	0·47
Pyruvic acid	0·00	0·00	0·00
Acetic acid	0·32	0·64	0·32
Ethanol	0·01	0·02	0·00
Sum	—	**5·83**	**2·67**
C g. atoms supplied (as glycogen and CO_2)	—	6·41	—
Acid yield (from bicarbonate destroyed)	—	—	2·74

Table 4. *Effect of inhibitors on anaerobic metabolism*

(Each manometer cup contained about 0·9 mg. ciliate N in a total vol. of 1·5 ml.; gas phase 5% CO_2–95% N_2; temp. 37°. Inhibitor added from side bulb, and CO_2 evolution over a period of 1 hr. compared with that in control.)

Inhibitor	Concn. (M)	CO_2 evolution (as % that of control)
Sodium fluoride	0·02	86
Sodium arsenite	0·01	66
Sodium iodoacetate	0·001	25
2:2′-Dipyridyl	0·0003	127
4:4′-Dipyridyl	0·0003	100
8-Hydroxyquinoline	0·0003	121
Phenylthiourea	0·0003	100

Intermediary carbohydrate metabolism

Amylase and maltase. Cell-free preparations of *T. pyriformis* (GL) were incubated with starch, glycogen or maltose at pH 7·3 in the presence of NaCl, and the reaction followed by estimating reducing sugar and glucose at suitable time intervals. From Table 5, it can be seen that starch and glycogen were hydrolysed at approximately the same rate to reducing sugar, 80% of which was found to be glucose. During the 90 min. incubation, 23·4 mg. maltose were broken down, and 24·8 mg. glucose were recovered. At the beginning of the experiment, the samples already contained a small amount of reducing sugar, due no doubt to hydrolysis of protozoal glycogen in the course of preparation of the cell-free extract.

Table 6. *Hexokinase activity of cell-free* Tetrahymena pyriformis *(GL) preparations*

(Each manometer cup contained 0·7 mg. N in the form of cell-free preparation, 0·026M-$NaHCO_3$, 0·005M-$MgSO_4$, 0·06M-NaF, 0·05M-KCl, 0·0045M-glucose and 0·0031M-ATP in a total volume of 2·5 ml. Mixture incubated 40 min. at 37° with gas phase of 5% CO_2–95% N_2.)

	Inorganic P $[P_0]$ (μmoles)	Acid-labile P $[P_7\text{-}P_0]$ (μmoles)	Reducing sugar (μmoles)
Initial	1·70	15·22	14·1
Final	11·81	4·01	14·5

P esterified = 1·10 μmoles. CO_2 liberated by acid production = 145 μl. (6·48 μmoles).

Hexokinase. An attempt was made to demonstrate hexokinase activity in cell-free preparations. Warburg manometers were set up as indicated in Table 6, the reaction being followed by the liberation of CO_2 due to acid formation. During the experiment, 145 μl. (6·48 μmoles) CO_2 were evolved; there was a large breakdown of ATP to inorganic phosphate, but about 10% was esterified. This was observed on two occasions. It was not possible to follow the hexokinase reaction by the disappearance of glucose since, during the experiment, there was a very slight increase in glucose, due no doubt to hydrolysis of polysaccharide present in the enzyme preparation. An identical system containing a similar homogenate of *Trypanosoma lewisi* showed marked hexokinase activity: 60% of the added glucose was utilized, and ATP was metabolized, equal amounts of inorganic and esterified P accumulating.

Table 5. *Amylase and maltase activity in cell-free* Tetrahymena pyriformis *(GL) preparations*

(Each tube contained 3·9 mg. N in the form of cell-free preparation, 0·027M-acetate-veronal buffer (pH 7·3), 0·017M-NaCl, and starch, glycogen and maltose respectively, in a total volume of 9 ml. Tubes incubated at 37°, 2 ml. samples being removed at intervals. Reducing sugar expressed in terms of mg. hexose. All figures given in mg.)

Incubation time (min.)		Starch	Glycogen	Maltose
0	Substrate added	36	36	60·2
	Reducing sugar found	2·02	2·03	35·6
	Glucose found	1·74	1·86	4·4
30	Reducing sugar found	6·85	6·41	40·0
90	Reducing sugar found	15·47	16·75	48·1
	Glucose found	12·35	13·26	29·2

Phosphorylase. Although *Tetrahymena pyriformis* (GL) contains hydrolytic enzymes capable of degrading glycogen and starch, in a system containing protozoal glycogen and phosphate buffer cell-free preparations of the ciliate caused a breakdown of glycogen, accompanied by an uptake of inorganic phosphate. During the first 15 min. of incubation, over 50% of the inorganic phosphate disappearing was converted to an easily hydrolysable P compound (Fig. 2). On further incubation this fraction decreased, while the less easily hydrolysable phosphate fraction increased. Fructose estimations in trichloroacetic acid extracts and in $Ba(OH)_2$-$ZnSO_4$ extracts showed that the process was accompanied by

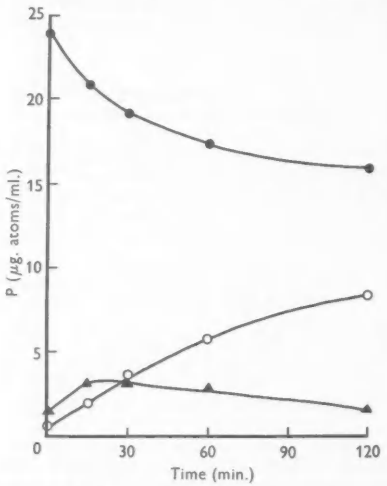

Fig. 2. Phosphorylase activity of cell-free *T. pyriformis* (GL) preparation. Each tube contained 0·85 mg./ml. N in the form of cell-free preparation, 4 mg./ml. *Tetrahymena* glycogen and 0·025 M-phosphate buffer (pH 7·3) in a total volume of 6 ml.; temp. 37°; ●—●, inorganic phosphate; ○—○, $[P_t\text{-}P_7]$ phosphate fraction; ▲—▲, $[P_7\text{-}P_0]$ phosphate fraction.

the formation of fructose phosphate. After 120 min. incubation, there had been an esterification of 7·6 μg. atoms/ml. P, and an accumulation of 2·7 μmoles/ml. fructose phosphate. In a similar experiment (Table 7), it was found that cell-free preparations of the ciliate were able to phosphorolyse starch as well as glycogen.

Phosphoglucomutase and oxoisomerase. The ability of *T. pyriformis* (GL) to transform glucose-1-phosphate into a mixture of hexose-6-phosphates has been shown directly by incubating Cori ester with a cell-free preparation of the ciliate. From Table 8 it can be seen that the easily hydrolysable phosphate fraction was reduced on incubation, 50% of the phosphate being recovered as inorganic phosphate, the other 50% being converted into less easily hydrolysable phosphate, part of it in combination with fructose, presumably as fructose-6-phosphate.

Table 7. *Phosphorylase activity of cell-free* Tetrahymena pyriformis *(GL) preparations*

(Each tube contained 0·5 mg./ml. N in the form of cell-free preparation, 4 mg./ml. starch or glycogen and 0·025 M-phosphate buffer (pH 7·3) in a total volume of 9 ml. Samples (2 ml.) deproteinized before and after 2 hr. incubation at 37°, with $Ba(OH)_2$-$ZnSO_4$ or with 5% trichloroacetic acid (TCA). Reducing sugar, glucose and fructose estimated in Ba-Zn filtrates, and fructose, P_0 and P_7 in TCA filtrates. Results expressed as μmoles/ml. of enzyme system.)

	Glycogen	Starch
Inorganic P esterified	5·36	3·55
Glucose-1-phosphate formed	1·29	0·42
Glucose-6-phosphate formed	1·85	1·81
Fructose-6-phosphate formed	2·22	1·42
Reducing sugar liberated	8·10	8·24
Free fructose found	0·16	0·22

Table 8. *Phosphoglucomutase and oxoisomerase activity in cell-free* Tetrahymena pyriformis *(GL) preparations*

(Each tube contained 1 mg. N in the form of cell-free preparation, 0·002 M-$MgCl_2$, 0·023 M-acetate-veronal buffer (pH 7·3) and 3 mg. potassium glucose-1-phosphate in a total volume of 4·5 ml. Incubation carried out at 37°, and systems deproteinized with 1·5 ml. 10% (w/v) TCA.)

Time (min.)	$[P_0]$ (μg. atoms)	$[P_7\text{-}P_0]$ (μg. atoms)	$[P_{180}\text{-}P_7]$ (μg. atoms)	$[P_t\text{-}P_{180}]$ (μg. atoms)
0	1·45	8·64	0·10	0·29
10	4·91	3·26	0·77	1·55
60	5·39	0·74	1·87	2·48

Glucose-1-phosphate used = 7·90 μmoles. Inorganic phosphate liberated = 3·94 μmoles. Less easily hydrolysable phosphate ester formed = 3·96 μmoles. Fructose-6-phosphate found (as fructose) = 2·77 μmoles.

Enzymic degradation of fructose diphosphate. Table 9 shows that a cell-free enzyme preparation from *T. pyriformis* (GL), incubated anaerobically with potassium fructose-1:6-diphosphate (FDP), gave rise to triosephosphate (alkali-labile phosphate); the conversion was greater in the presence of cyanide, which acts as a keto fixative. Using a similar

Table 9. *Aldolase activity in cell-free* Tetrahymena pyriformis *(GL) preparations*

(Each manometer cup contained 0·4 mg. N in the form of cell-free preparation, 0·025 M-$NaHCO_3$, 0·06 M-NaF and 0·01 M-fructose-1:6-diphosphate (FDP) in a total volume of 2·5 ml.; gas phase 5% CO_2–95% N_2; temp. 37°. Mixtures incubated for 1 hr., and deproteinized with 1·5 ml. 10% TCA.)

System	Alkali-labile P formed (μmoles)	FDP used (μmoles)	Inorganic P liberated (μmoles)
Enzyme + FDP	8·95	6·35	1·3
Enzyme + FDP + 0·06 M-KCN	21·10	13·20	1·0

system, it was found that 0·001 M-2:2′dipyridyl had no effect on aldolase activity at pH 7·3. However, at pH 6·0, aldolase activity was inhibited by 5% in the presence of 0·001 M-2:2′-dipyridyl, and by 34% in the presence of 0·005 M-dipyridyl.

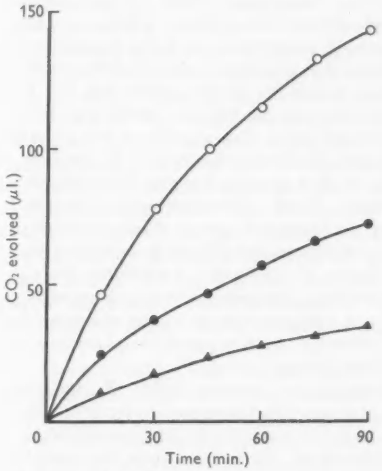

Fig. 3. Fructose diphosphate metabolism by cell-free *T. pyriformis* (GL) preparation. Each manometer cup contained 0·73 mg. N in the form of cell-free preparation, 0·025 M-$NaHCO_3$, 0·008 M-fructose-1:6-diphosphate(FDP), 0·008 M-pyruvate, 0·0001 M-cozymase, 0·0018 M-phosphate and 0·06 M-NaF in a total vol. of 2·5 ml.; gas phase 5% CO_2–95% N_2; temp. 37°; ●—●, CO_2 evolution (μl.) in presence of FDP; ○—○, CO_2 evolution (μl.) in presence of FDP and 0·004 M-arsenate; ▲—▲, CO_2 evolution (μl.) in absence of FDP.

Speck & Evans (1945), using malaria parasite preparations, attempted to demonstrate the oxidation of triosephosphate, coupled with the simultaneous reduction of pyruvate, fluoride being added to the system in order to prevent the breakdown of phosphoglyceric acid. A similar system has been used in the present case of *T. pyriformis* (GL). Fig. 3 illustrates an experiment in which a cell-free preparation of the ciliate was allowed to act upon FDP under anaerobic conditions, acid production being followed manometrically. Even in the absence of FDP, appreciable amounts of acid were formed, owing to the fermentation of some substrate present in the enzyme preparation itself; however, when FDP was added, CO_2 evolution was doubled.

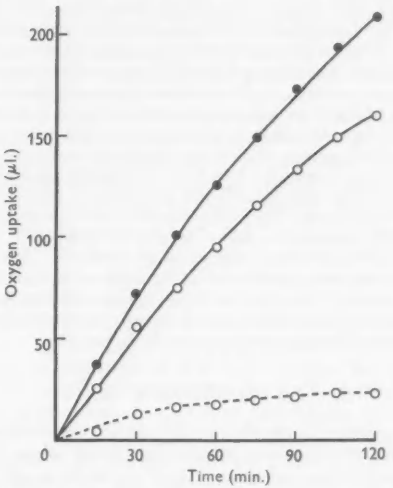

Fig. 4. Lactic oxidase activity of cell-free *T. pyriformis* (GL) preparation. Each manometer cup contained 1·0 mg. N in the form of cell-free preparation, and 0·04 M-phosphate buffer (pH 7·3), in a total volume of 2·5 ml.; temp. 37°; gas phase air; ●—●, O_2 uptake (μl.) in presence of 0·03 M-lactate; ○—○, O_2 uptake (μl.) in presence of 0·03 M-lactate and 0·00005 M-methylene blue; ○- - - -○, O_2 uptake (μl.) in presence of 0·00005 M-methylene blue.

In the presence of FDP, fermentation proceeded at the same rate in the presence as in the absence of added pyruvate or cozymase. Chemical analysis showed that at the end of this experiment about 40% of the added FDP had disappeared; triosephosphate formation accounted for 60% of the FDP disappearing, while the remainder was converted to lactic acid. The production of lactic acid was not influenced by the addition of extra pyruvate although, when present, pyruvate was utilized. Arsenate (0·004 M) doubled the rate of acid production in the presence of FDP, and there was a corresponding increase in FDP utilization. Similar experiments

Table 10. *Dehydrogenase activity in* Tetrahymena pyriformis *(GL)*

(Thunberg tubes contained homogenate or cell-free preparation and 0·04 M-phosphate buffer (pH 7·3), with 0·134 μmole methylene blue and substrate as indicated added from hollow stopper. Tubes evacuated and washed with pure N_2 four times; temp. 37°; total volume, 2·5 ml. Figures give time (min.) for 90% decolorization.)

	Additions				
Preparation	None	Lithium lactate (0·03 M)	Sodium succinate (0·03 M)	Glucose (0·03 M)	FDP (0·008 M)
Homogenate (2 mg. N)	45	33	11	37	—
Homogenate (3·9 mg. N)	33	27	8·5	23	—
Cell-free preparation (1·8 mg. N)	80	37	25	—	—
Cell-free preparation (1·1 mg. N)	150	65	37	—	61

showed that the endogenous fermentation of the preparation was insensitive to iodoacetate, but that acid formation in the presence of FDP, with or without arsenate, was reduced to the endogenous level by the addition of 0·001 M-iodoacetate.

As already stated, during glycogen fermentation by intact cells of *T. pyriformis* (GL), lactic and succinic acids accumulate. It seems probable that the production of these acids is a means of reoxidizing dihydrocozymase, which is formed from cozymase during the oxidation of phosphoglyceraldehyde. Table 10 gives the results obtained from a number of Thunberg tube experiments, using both whole and centrifuged homogenates of the ciliate. Succinate accelerated the reduction of methylene blue fourfold, while lactate, glucose and fructose diphosphate were less effective. The centrifuged preparation alone did not respire, but in the presence of 0·03 M-lactate, there was a vigorous O_2 uptake ($q_{O_2} = 125$; Fig. 4). On the other hand, succinate, which was shown in Thunberg tube experiments to accelerate methylene blue reduction, did not increase the O_2 uptake of homogenates when tested manometrically, either in the presence or in the absence of added cytochrome *c* or methylene blue.

DISCUSSION

The ciliate *T. pyriformis* (GL) is able to maintain active motility and respiration in the absence of extracellular substrates over a wide range of temperature, in contrast to the trypanosomes, flagellates which have become adapted to life in a specific and constant environment on which they rely for their source of energy. *T. pyriformis* (GL) respires with a q_{O_2} of 480 at 37°, and 225 at 25°. The nature of the intracellular substrates responsible for this endogenous respiration is an interesting matter for speculation. The present study indicates that respiration is relatively independent of the intracellular glycogen reserve. A respiratory quotient of 0·85 suggests that the ciliates may be oxidizing fat or protein, rather than carbohydrate. Pace & Ireland (1945) demonstrated the presence of fat droplets in the ciliate by staining techniques, while Wilber & Seaman (1948) showed that the organisms contain lipid, and a small amount of phospholipin. However, Pace & Lyman (1947) were unable by microscopical methods to observe any utilization of fat during the metabolism of *T. pyriformis* (W); it would be interesting to confirm this, carrying out chemical estimations of cell lipid before and after a period of active respiration.

Although *T. pyriformis* (GL) respires well in the absence of extracellular substrates, a number of compounds are able to increase the oxygen uptake. Chaix *et al.* (1947) found that the endogenous respiration of this strain was stimulated by short-chain fatty acids, but was little affected by citrate, succinate or malate, substances associated with the tricarboxylic acid cycle. The present study confirms these observations, except that the respiratory stimulations produced by fatty acids were larger; this may well be due to the higher temperature used in the present work. In contrast to these findings, Seaman (1949) reported that the endogenous respiration of the S strain of *T. pyriformis* was doubled in the presence of 0·02 M-succinate, α-ketoglutarate, fumarate, malate or oxaloacetate, although citrate was without effect, and that the increase in respiration produced by pyruvate was reversed by malonate, this malonate inhibition being antagonized by fumarate. On the basis of these observations, Seaman (1949) suggested that respiration in the ciliate involved the tricarboxylic acid cycle. With the GL strain of *T. pyriformis* used in the present study it has not been possible to obtain such effects. Moreover, unlike Seaman (1950, 1951), we could also find no evidence for the oxidation of succinate by cell homogenates prepared from the ciliate. No oxidation of succinate was observed with cell homogenates or particle-free preparations, either in the presence or in the absence of added cytochrome *c*; such preparations, however, readily oxidized lactate.

According to Seaman (1950), *T. pyriformis* (S) also attacks added acetate, partly by oxidizing it, and partly by converting it to intracellular lipid and carbohydrate. However, since his carbohydrate analyses were carried out on filtrates prepared from cell suspensions deproteinized with barium hydroxide and zinc sulphate, it is difficult to assess the relation between these results and the total carbohydrate content of the cells. It would appear from Seaman's data, that the initial 'carbohydrate' content of the cells was of the order of 0·1 %, a value which contrasts with a content of 20 % glycogen demonstrated by us in the case of *T. pyriformis* (GL) (Ryley, 1951; Manners & Ryley, 1952).

In the present study, it was found that the respiration of *T. pyriformis* (GL) was not affected by the presence of carbon monoxide ($CO/O_2 = 19$) in the gas phase, and that cyanide at a concentration of $0{\cdot}46 \times 10^{-3}$ M inhibited endogenous respiration by only 18 %. Lwoff (1934) found that respiration of the GL strain in a peptone solution was cyanide-sensitive, but that in Ringer-glucose solution, 0·0022 M-cyanide stimulated respiration by 36 %. Using the H strain of *T. pyriformis*, Hall (1941) observed that respiration was inhibited 88 % by 0·001 M-cyanide, or 35 % by 0·0001 M-cyanide. Baker & Baumberger (1941), using the T strain of *T. pyriformis*, observed that respiration was blocked, and oxidation of the cytochrome bands prevented, in the presence of an unspecified concentration of cyanide. They also found that respiration was sensitive to carbon monoxide, but this effect was only observed at very high partial pressures of carbon monoxide ($CO/O_2 = 45$). It would seem that on the whole, while cyanide probably has some effect on the respiration of *T.*

pyriformis, the cyanide-sensitivity of the process is far below that of other cells with typical cytochrome oxidase activity. These facts, and the absence of any *p*-phenylenediamine oxidase activity in cell homogenates, are in agreement with the spectroscopic observations reported. We observed very little absorption at 605 mμ., the location of cytochrome *a*, usually involved in cyanide-sensitive respiration, but in contrast to this there was intense absorption in the green part of the spectrum. This was considered to be due to a strong cytochrome *e* band, with a weaker *b* band, and a weak *c* band. These results are in good agreement with the spectroscopic observations of Baker & Baumberger (1941) on the T strain, but they attributed their band at 552 mμ. to cytochrome *c*, rather than to cytochrome *e*. They found absorption in the region 587–592 mμ., and in some cases at 616–617 mμ., but they were not able to observe the α-band of oxyhaemoglobin, which appears at about 582 mμ. under conditions of good aeration.

It has been noted (Experimental section) that glucose stimulated respiration to a small extent, and that under aerobic conditions appreciable amounts of sugar were removed from the medium. Kidder & Dewey (1945) noted that most strains of *T. pyriformis* examined were able to ferment glucose, fructose, mannose, maltose, starch, dextrin and glycogen, but no other carbohydrates tested. Niel *et al.* (1942) examined the glucose metabolism of the T strain under anaerobic conditions, and showed qualitatively the production of succinic, lactic and acetic acids. They were able, by the use of radioactive carbon, to demonstrate the assimilation of carbon dioxide, and its incorporation in the carboxyl groups of succinic acid. The high intracellular carbohydrate content of *Tetrahymena* appears to have escaped the notice of all previous investigators, nor have they realized the significance of the intracellular carbohydrate reserve for the anaerobic metabolism of the ciliate. The present study shows that under anaerobic conditions, the ciliate *T. pyriformis* (GL) maintains a normal degree of motility at the expense of its vast glycogen store. At room temperature, under strictly anaerobic conditions, the ciliate maintained normal motility in the absence of any extracellular nutrient for at least 72 hr. Although it seems doubtful that the ciliate will reproduce under anaerobic conditions, yet, contrary to the findings of Pace & Ireland (1945) with the W strain, the organism will survive. It is interesting to compare these ciliates with the haemoflagellate *Trypanosoma lewisi* (Ryley, 1951); the latter organism is able to survive in the presence of extracellular substrate under anaerobic conditions, but motility is much less than that under aerobic conditions. With *Tetrahymena pyriformis* (GL), motility under anaerobic conditions is just as vigorous as under aerobic conditions. There is a striking similarity between the anaerobic metabolic processes of the two organisms; in the trypanosome, extracellular glucose is fermented to a mixture of succinic, lactic and acetic acids, with the simultaneous assimilation of carbon dioxide; with the ciliate, intracellular glycogen is fermented with the formation of exactly the same products.

In cell-free preparations of the ciliate, the presence of phosphorylase, phosphoglucomutase and oxoisomerase has been demonstrated. The phosphorylase of the ciliate acts upon starch as well as glycogen. Hydrolytic enzymes capable of degrading these polysaccharides have also been demonstrated. Cell-free preparations of *T. pyriformis* (GL) readily convert fructose diphosphate to triosephosphate, by an enzyme system which does not seem to be sensitive towards 2:2′-dipyridyl. In this respect, the aldolase of the ciliate resembles the animal, rather than the yeast type of enzyme (Warburg & Christian, 1943). The occurrence of phosphorylative mechanisms in carbohydrate breakdown by *T. pyriformis* (GL) is also supported by the observations relating to the formation of lactic acid from fructose diphosphate by cell-free enzyme preparations, the stimulation of this process by arsenate, and its inhibition by iodoacetate.

SUMMARY

1. The free-living ciliate *Tetrahymena pyriformis* (*Glaucoma piriformis*) maintains metabolism and motility in the absence of extracellular nutrients for considerable periods of time at room temperature or at 37°.

2. Under aerobic conditions at 37°, the ciliate respires with a q_{O_2} of 480 and an R.Q. of 0·85. This endogenous respiration is practically independent of the intracellular glycogen, is only slightly stimulated by added glucose and a number of dicarboxylic acids, but is almost doubled by 0·01 M-acetate, propionate or butyrate.

3. High concentrations of azide or 2:4-dinitrophenol inhibit endogenous respiration, while low concentrations stimulate it; in all cases, motility in the presence of these substances is reduced.

4. The cytochrome system of the ciliate is composed of cytochromes *b*, *c* and *e*, but very little *a*; homogenates display no cytochrome oxidase activity towards *p*-phenylenediamine. Respiration of the ciliate is comparatively resistant to cyanide and carbon monoxide.

5. *T. pyriformis* contains a heat-labile peroxidase (purpurogallin number, 0·033); in the presence of hydrogen peroxide this peroxidase is able to oxidize a number of phenolic compounds, and reduced cytochrome *c*.

6. Anaerobic metabolism and motility depend on the fermentation of the intracellular glycogen; in

the absence of extraneous carbohydrate, the ciliate produces large amounts of succinic acid, with smaller quantities of lactic and acetic acid, and assimilates some carbon dioxide.

7. Cell-free, centrifuged homogenates contain enzymes capable of hydrolysing starch, glycogen and maltose to glucose; they are also able to phosphorylate glycogen and starch to form glucose-1-phosphate and contain phosphoglucomutase and oxoisomerase.

8. Cell-free preparations convert fructose-1:6-diphosphate to triosephosphate, which is fermented to lactic acid. This process is stimulated by arsenate, and inhibited by iodoacetate.

9. Homogenates of the ciliate display both succinic and lactic dehydrogenase activity; in the presence of atmospheric oxygen, however, they oxidize lactic, but not succinic, acid.

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Glyoxalase: the Role of the Components

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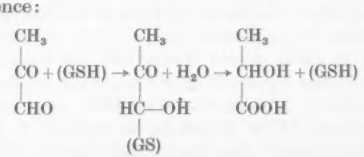
Since the discovery by Lohmann (1932) that reduced glutathione (GSH) is the coenzyme of glyoxalase, several workers have reported that methylglyoxal and GSH form a compound and have suggested that this might be the true substrate for the enzyme or an intermediate in the reaction. Thus Jowett & Quastel (1932) observed that SH groups titratable by iodine decreased when methylglyoxal was added to GSH solution and suggested a reaction sequence:



Platt & Schroeder (1934) studied this interaction of methylglyoxal and GSH, showing that under the usual conditions of temperature, pH and concentration of reagents, 80% of the GSH was combined. However, they reported, as had most earlier observers, that the rate of transformation of methylglyoxal was constant until the reaction was almost complete. They commented upon the difficulty of reconciling the two observations, since, if the methylglyoxal-GSH compound were the substrate for the enzyme and its formation were only 80% complete at the initial concentration of the reagents, the reaction rate would be expected to fall off from the beginning of the reaction. Giršavičius & Heyfetz (1935) created further difficulties when they showed that addition of enzyme to the equilibrium mixture of methylglyoxal and GSH caused a further decrease of SH concentration almost to zero, and that this low figure was maintained until the reaction was almost complete, when the whole of the GSH rapidly reappeared. They suggested that the enzyme drove the combination of methylglyoxal and GSH to completion, a concept entirely out of harmony with our present ideas of the role of enzymes as catalysts. The problem was clarified by Yamazoye's (1936) demonstration that the compound formed from methylglyoxal and GSH in the presence of rabbit-liver glyoxalase was different from that formed in its absence. He was able to isolate both. The compound formed in the absence of enzyme was very labile and behaved as a mixture of GSH and methylglyoxal. This, Yamazoye called the 'Chemical compound' and its properties are closely similar to those of the compounds described by Schubert (1935). The 'Biological compound', formed in the presence of the enzyme, did not react with iodine and gave no tests for SH, keto or aldehyde groups. Both compounds, however, contained GSH and methylglyoxal in equimolecular proportions. The biological compound, above pH 7·0, slowly broke down to form GSH and lactic acid. Yamazoye was able to show that this breakdown was catalysed by his crude rabbit-liver glyoxalase.

Later, Hopkins & Morgan (1948) demonstrated that ox-heart glyoxalase consisted of two components. One of these, called by them the 'enzyme', in the presence of GSH, converted methyl- and phenylglyoxals to the corresponding hydroxy acids at equal rates. The second component they called the 'factor' and this, when added to the system, accelerated the conversion of methylglyoxal but did not affect the rate of conversion of phenylglyoxal. In the absence of 'enzyme', the 'factor' had no effect on either glyoxal. Hopkins & Morgan (1948) also showed that phenylglyoxal inhibited the accelerating effect of the 'factor' upon the conversion of methylglyoxal. They suggested that this was due to combination of phenylglyoxal with the 'factor' thereby preventing it from acting.

It seemed possible that the results of Hopkins & Morgan might be most easily interpreted by assuming that their 'enzyme' was responsible for catalysing the formation of Yamazoye's 'biological compound' of methylglyoxal and GSH. As he had shown this to break down slowly into lactic acid and GSH, the fairly slow activity of the 'enzyme' would be explained. Further, as Yamazoye had shown that crude glyoxalase accelerated this breakdown of his intermediate, it seemed likely that there was another enzyme present in crude preparations, and that Hopkins & Morgan had separated this and called it 'factor'. The difference in behaviour of methyl- and phenylglyoxals with the 'factor' could then be explained in terms of the specificity of this second enzyme. In this paper evidence for the correctness of this hypothesis is presented. A preliminary account of this work has been given (Crook & Law, 1950), and Racker (1950, 1951) has described similar experiments carried out independently.

EXPERIMENTAL

Enzymes. Acetone-dehydrated ox-heart powder, prepared and extracted by the method of Hopkins & Morgan (1948), was used as a source of both 'enzyme' and 'factor'. The 'enzyme' was purified by a method worked out by Dixon & Morgan (1948) and 'factor' was prepared according to Hopkins & Morgan (1948). 'Factor' was considered to be free from 'enzyme' when there was no reaction above the controls. The criteria for judging freedom of the 'enzyme' from 'factor' are much less certain. Arbitrarily it was decided to regard the 'enzyme' as free from 'factor' when the rates of conversion of equimolecular concentrations of methyl- and phenylglyoxal were equal. In this paper the nomenclature of Hopkins & Morgan (1948) will be used and inverted commas will not be used from now on.

Preparation of the glyoxal-GSH compounds. Chemical and biological compounds were prepared in the solid state by the methods of Yamazoye (1936). In certain experiments small quantities of biological compound were prepared in solution by allowing the enzyme to act upon a mixture of appropriate quantities of the glyoxal and GSH for approximately 20 min., precipitating the enzyme with sufficient 10% (w/v) salicylsulphonic or trichloroacetic acid to bring the pH to 2·0, filtering off the precipitate and readjusting the pH to 7·3. Solutions were always used at once since the biological compound is unstable at the higher pH.

Reaction rate. This was measured manometrically in Warburg manometers at 25° following the technique of Hopkins & Morgan (1948). The flasks contained 0·4 ml. of 0·2 M-$NaHCO_3$ solution, water and enzymes. In the side arm were placed 0·3 ml. containing 2·5 mg. of GSH and 0·3 ml. containing 2·1 mg. of methylglyoxal or 4·0 mg. of phenylglyoxal, adjusted to pH 7·3. The total volume was 2·0 ml. The flasks were gassed at room temperature with 95% N_2–5% CO_2 (v/v) for 10 min. The reaction rate was measured between 5 and 25 min.

Rate measurements were also carried out by following the change in SH groups by iodine titration. Final concentra-

tions of reagents and the temperature were the same as for the manometric experiments. Initial samples were taken immediately after addition of enzyme. Samples (2 ml.) were removed at suitable intervals into 2 ml. of 10% (w/v) trichloroacetic or 20% (w/v) salicylsulphonic acid solution; 5 ml. of 0·0025 N-I_2 were then added, the solution allowed to stand for 3 min. for any chemical compound between the glyoxal and GSH to react with the iodine, and the excess iodine back-titrated with 0·0025 N-$Na_2S_2O_3$ using starch as indicator.

RESULTS

Role of enzyme and factor. Fig. 1 (upper part) shows the course of the liberation of CO_2 from bicarbonate when crude acetone heart-powder extract acts upon methyl- or phenylglyoxal. With methylglyoxal the reaction is rapid (325 μl. CO_2 in 20 min.) and is practically complete in half an hour. With phenylglyoxal the reaction is much slower (150 μl. CO_2 in 20 min.) and, although it will finally go to completion, may require several hours. In the lower portion of Fig. 1 is shown the simultaneous changes in the concentration of SH groups expressed as mg. GSH in 20 ml. of reaction mixture. With both the glyoxals the concentration of GSH falls rapidly and to approximately the same extent in the initial stages of the reaction. The subsequent behaviour is different. With methylglyoxal the reaction follows the course described by Yamazoye (1936), the GSH rapidly reappearing and reaching a value very little lower than the initial value at approximately the same time as the evolution of CO_2 ceases, i.e. when all the methylglyoxal has been converted into lactic acid. With phenylglyoxal the behaviour of the GSH is quite different. It remains at a low concentration and may even go on decreasing for periods up to 1 hr. It is at this stage that the long-continuing slow output of CO_2 is observed in the manometric experiments.

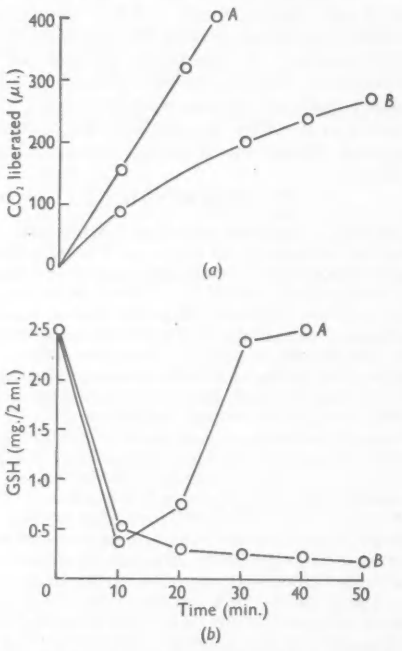


Fig. 1. (*a*) Liberation of CO_2 from 0·2 M-bicarbonate buffer by extract of heart acetone powder (9 mg. dry matter), using 2·5 mg. GSH with 2·1 mg. methylglyoxal (curve *A*), and with 4 mg. phenylglyoxal (curve *B*) in a total vol. of 2 ml. at 25°. (*b*) Uptake and liberation of GSH by extract of heart acetone powder (90 mg. dry matter) with methylglyoxal (curve *A*) and phenylglyoxal (curve *B*). The amounts used were 10 times those of the manometric experiment in a total vol. of 20 ml.

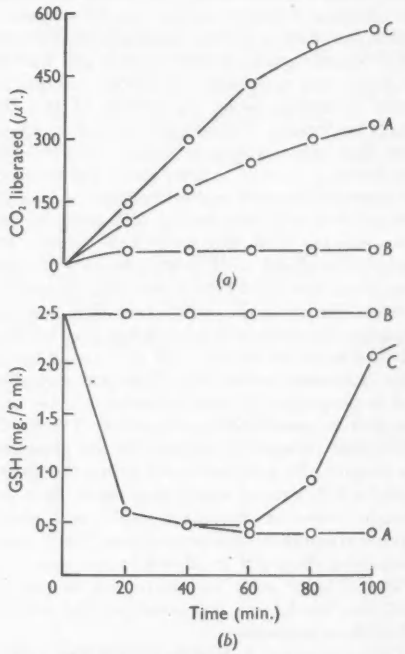


Fig. 2. (*a*) Liberation of CO_2 from 0·2 M-bicarbonate buffer by 0·75 mg. purified enzyme (*A*), 0·5 mg. purified factor (*B*) and enzyme + factor (*C*), using 2·1 mg. methylglyoxal as substrate with 2·5 mg. GSH in a total vol. of 2 ml. at 25°. (*b*) Uptake and liberation of GSH by 3 mg. purified enzyme (*A*), 7 mg. purified factor (*B*), and enzyme + factor (*C*) with methylglyoxal as substrate. The amounts of all components were 10 times those used in the manometric experiment.

Fig. 2 shows the results of similar experiments with methylglyoxal and purified enzyme and factor. Factor alone is inert both in the production of CO_2 and in causing a disappearance of SH groups. Pure enzyme in the absence of factor behaves with methylglyoxal in a manner very similar to the behaviour of the crude extract with phenylglyoxal. The CO_2 output is slow and long continued and the GSH concentration decreases rapidly to a low value and remains thus for many hours. In one experiment it remained low for 5 hr. and had not regained its initial concentration for 10 hr. However, when

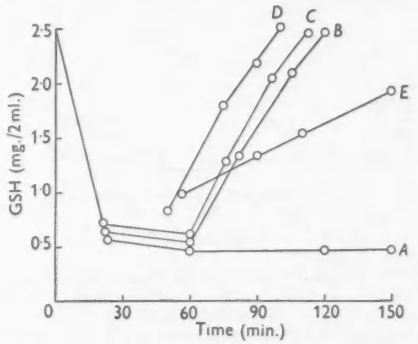


Fig. 3. The formation and breakdown of the methylglyoxal-GSH compound by enzyme and factor. GSH, 25 mg.; methylglyoxal, 21 mg.; buffer and enzyme in an initial vol. of 20 ml., incubated at 25°. (*A*) Action of 2 mg. enzyme alone; (*B*) action of 2 mg. enzyme + 3 mg. factor added initially; (*C*) addition of factor after 40 min.; (*D*) enzyme precipitated with salicylsulphonic acid after 20 min., pH readjusted to 7·3 and factor added; (*E*) enzyme precipitated after 20 min., pH readjusted to 7·3—no addition of factor.

enzyme and factor are mixed the properties of Fig. 1 are restored and the familiar U-shaped curve is observed.

For the experiment shown in Fig. 3, purified factor and purified enzyme were used. Curves *A* and *B* are given by enzyme alone and by enzyme + factor and follow the same course as before. For curve *C*, the experiment was started with enzyme alone but, after the GSH concentration had fallen to its lowest value, factor was added. Curve *D* is obtained if the enzyme is precipitated after 20 min. and factor added. This experiment shows that the factor always has the effect of liberating GSH from combination whether it be added with the enzyme, some time later, or after the enzyme has been removed. On the other hand, the spontaneous breakdown of the glyoxal-GSH compound is quite slow (curve *E*).

Specificity of factor. The behaviour of the phenyl- and methylglyoxal compounds with factor is shown in Fig. 4. For this experiment the compounds were prepared in solution. It will be seen that in the absence of factor GSH is liberated quite slowly from each (curves *A* and *B*). The rate of liberation from the phenyl compound is little affected by the addition of factor (curve *C*), whereas the rate of liberation from the methylglyoxal compound is much increased (curve *D*). A similar result was obtained manometrically. The results are shown in Table 1.

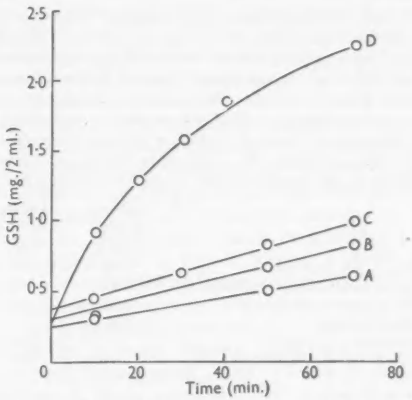


Fig. 4. Specificity of factor. 21 mg. methylglyoxal or 40 mg. phenylglyoxal + 25 mg. GSH incubated at 25° with 2 mg. enzyme in buffer, the total vol. being 5 ml. After 20 min., enzyme precipitated with salicylsulphonic acid, the pH readjusted to 7·3 and the solution diluted 3·3 times (equivalent to that in the manometric experiment, Table 1). (*A*) Methylgloxal as substrate—no addition after precipitation of enzyme; (*B*) phenylglyoxal as substrate—no addition after precipitation of enzyme; (*C*) phenylglyoxal as substrate—addition of 3 mg. factor after precipitation of enzyme; (*D*) methylglyoxal as substrate—addition of 3 mg. factor after precipitation of enzyme.

Table 1. *Manometric demonstration of the specificity of factor using the intermediate compounds formed between glutathione and phenyl- and methylglyoxal*

(21 mg. methylglyoxal or 40 mg. phenylglyoxal and 2 ml. 0·2 M-$NaHCO_3$ + 25 mg. GSH incubated with enzyme, total vol. 5 ml. After 20 min. the enzyme precipitated with salicylsulphonic acid, and the pH readjusted to 7·3. 0·6 ml. of the solution measured into the side arm of the Warburg flask, factor and bicarbonate being in the main compartment; total vol. 2 ml.)

	Evolution of CO_2 (μl.)			
Time (min.)	Phenylglyoxal compound alone	Phenylglyoxal compound + factor	Methylglyoxal compound alone	Methylglyoxal compound + factor
10	49	52	40	80
20	72	79	59	119
30	84	94	72	145

Composition of the intermediate compound. Yamazoye's (1936) finding that this contains equimolecular amounts of glyoxal and GSH indicates that it should give rise to equimolecular amounts of GSH and lactic acid on decomposition.

Table 2. *Equivalence of the reduced glutathione (GSH) and lactic acid (CO_2) liberated on decomposition of the intermediate compound of methylglyoxal and reduced glutathione*

(In each Warburg flask 4 mg. biological compound prepared from methylglyoxal by the method of Yamazoye (1936) together with 0·4 ml. 0·2 M-$NaHCO_3$ and water to total vol. 2·0 ml. In side arms 0·6 mg. of ox-heart factor in 0·6 ml. water. Controls without factor. Gassed at room temperature; reaction at 25°. Theoretical CO_2 calculated on SH liberated on basis of 1 equiv. of CO_2 per equivalent of SH. Experimental CO_2 represents difference between flasks with and without factor. Control CO_2 output 6–7 μl.)

	Amount of GSH (mg.)	Amount of CO_2 (μl.)
Initial GSH content	1·06	—
GSH in control after reaction	0·94	—
GSH in flask with factor	2·41	—
GSH liberated	1·5	—
Theoretical CO_2 equiv.	—	110
Experimental CO_2 found	—	111

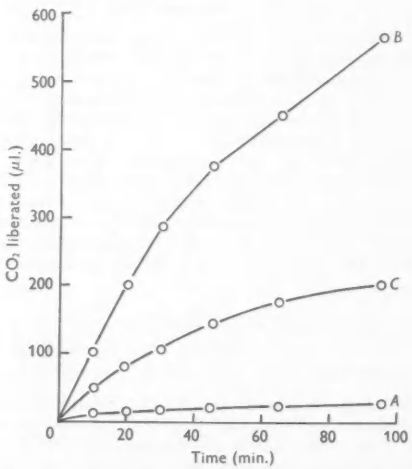


Fig. 5. Action of purified and crude enzyme on the chemical compound of methylglyoxal and GSH. CO_2 output at 25° from 0·2 M-bicarbonate buffer and 10 mg. chemical compound in the presence of 0·3 mg. pure glyoxalase enzyme (curve *A*), 0·3 mg. pure enzyme + 5 mg. methylglyoxal (curve *B*) and crude extract of heart acetone powder containing 9 mg. dry matter (curve *C*). Total vol. in each vessel 1·5 ml.

To check this, 4 mg. quantities of biological compound of methylglyoxal and GSH were placed in pairs of Warburg vessels together with bicarbonate. One flask of each pair contained 0·6 mg. factor purified to stage 6 of Hopkins & Morgan (1948); the other served as control. The liberation of CO_2 was followed until it had ceased (after 15 min.). The contents of each flask were then washed out, acidified with salicylsulphonic acid and the GSH titrated with iodine.

The compound contained approximately 20 % of free GSH, and this was usually found not to have increased in the controls, i.e. there was little spontaneous breakdown of the compound. The results of a typical experiment are shown in Table 2.

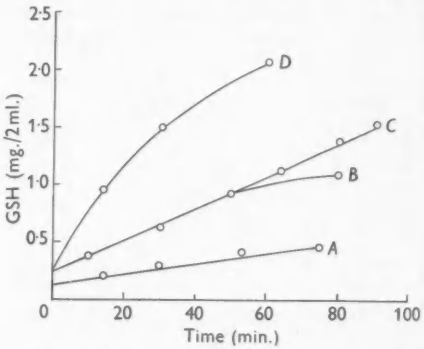


Fig. 6. Effect of extra methyl- or phenylglyoxal on the breakdown of the methylglyoxal compound. Methylglyoxal, 21 mg.; GSH, 25 mg.; buffer and 3 mg. enzyme in a total vol. of 20 ml. were incubated for 20 min. at 25°. The enzyme was precipitated with salicylsulphonic acid, and the pH readjusted to 7·3. (*A*) The methylglyoxal-GSH compound without any addition; (*B*) 21 mg. methylglyoxal added to the compound; (*C*) 40 mg. phenylglyoxal added to the compound; (*D*) 3 mg. factor added to the compound.

Effect of methyl- and phenylglyoxal on the biological compound. Yamazoye (1936) has shown that the chemical compound when investigated by chemical tests behaves as though it were simply a mixture of methylglyoxal and GSH. It would therefore be expected to react with factor-free enzyme as would any other mixture of methylglyoxal and GSH, i.e. a slow but steady reaction. However, Fig. 5, curve *A*, shows that this is not so. The system is inert. However, when extra methylglyoxal is added, reactivity is normal (curve *B*). The presence of factor (curve *C*) also renders the system reactive even in the absence of extra methylglyoxal. The accelerating effect of extra methylglyoxal is exerted upon the breakdown of the biological compound as is shown in Fig. 6. Here the compound was prepared in solution from the usual initial concentrations of reagents and enzyme, and the enzyme precipitated after 20 min. Curve *A* shows the rate of spontaneous liberation of GSH in the presence of

0·75 mg. methylglyoxal per ml., i.e. the concentration usually remaining when the intermediate compound is made in this way. Curves *B* and *C* show the effects of adding 1 mg. of methyl- and 2 mg. of phenylglyoxal per ml. The reaction rate has been increased approximately threefold in each case.

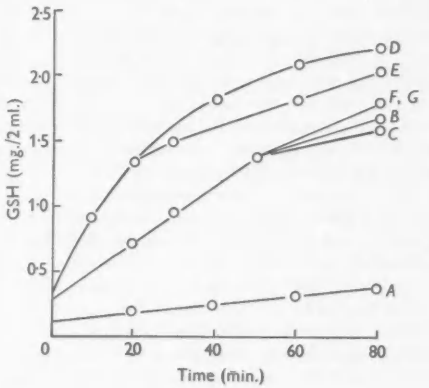


Fig. 7. Experiment to show the point in the reaction sequence where phenylglyoxal inhibits the acceleration by factor of the conversion of methylglyoxal to lactic acid. 25 mg. GSH, 21 mg. methylglyoxal, and 40 mg. phenylglyoxal added as shown above. Initial volumes, 20 ml., contained 4 ml. 0·2 M-$NaHCO_3$ + purified enzyme and were incubated for 20 min. at 25°. Enzyme pptd. with 10% (w/v) salicylsulphonic acid, pH adjusted to 7·3, 2 ml. 0·2 M-$NaHCO_3$ added, and addition of factor and phenylglyoxal made, as indicated. All volumes adjusted to be equal, by addition of water where necessary.

Curve	Material incubated with enzyme	Material added after precipitation of enzyme
A	GSH + methylglyoxal	Nil
B	GSH + methylglyoxal	Methylglyoxal
C	GSH + methylglyoxal	Phenylglyoxal
D	GSH + methylglyoxal	Factor
E	GSH + methylglyoxal	Factor + phenylglyoxal
F	GSH + methylglyoxal + phenylglyoxal	Nil
G	GSH + methylglyoxal + phenylglyoxal	Factor

Curve *D* shows the effect of adding factor. It is thus clear that extra amounts of these glyoxals accelerate the overall reaction by increasing the rate of breakdown of the intermediate compounds. Thus, although the reaction rate may increase with GSH concentration in the lower ranges of concentration of these compounds as has been found by many authors, there will be a tendency for the reaction to slow down as a unimolecular ratio of GSH to glyoxal is approached. Such an effect presumably explains the well known fact that GSH becomes inhibitory beyond a certain critical concentration (Platt & Shroeder, 1934).

Inhibition by phenylglyoxal. Phenylglyoxal inhibits the accelerating effect of factor upon the conversion of methylglyoxal to lactic acid. Hopkins & Morgan (1948) ascribed this to combination of the phenylglyoxal with the factor thus preventing it from acting. The present experiments show that factor does not act directly upon either of the glyoxals, nor upon the intermediate compound formed by the enzyme between phenylglyoxal and GSH, but only upon the intermediate between methylglyoxal and GSH. Phenylglyoxal could therefore exert its inhibitory effect not only by combining with factor but also by competing for the available GSH, thus preventing the formation of a compound susceptible to the action of factor. These possibilities were tested experimentally.

The intermediate compound of methylglyoxal was prepared in the presence and in the absence of phenylglyoxal and the subsequent liberation of SH groups from it was followed after removal of the enzyme by trichloroacetic acid precipitation. The results are shown in Fig. 7. Curve *A* shows the usual slow spontaneous liberation of SH from the intermediate compound, while curves *B* and *C* show how this is accelerated by increase in the concentration of the glyoxals. Methyl- and phenylglyoxal have equal effects when added in equivalent concentrations. From curve *D* it can be seen that the effect of factor is much greater than that of the extra glyoxals and is not markedly inhibited by the presence of phenylglyoxal (curve *E*). However, when phenylglyoxal was present in the initial stages of the reaction, when the enzyme is acting, the intermediate compound has quite different properties. Although its rate of spontaneous breakdown is unchanged (curve *F*) and is fast due to the high total methyl or phenylglyoxal concentration, this rate is unaffected by the addition of factor (curve *G*), a property which in these experiments has been associated with the phenylglyoxal-GSH intermediate compound. Thus in the presence of both glyoxals it is the phenylglyoxal-GSH intermediate which is preferentially formed. As the breakdown of this compound is not accelerated by factor, the inhibitory effect of phenylglyoxal found by Hopkins & Morgan (1948) is understandable.

DISCUSSION

There seems little doubt that the conversion of glyoxals to the corresponding hydroxy acids by the addition of one equivalent of water under the influence of glyoxalase proceeds by way of an intermediate compound with glutathione. The relationships between the two catalysts involved also seems clear. That named 'glyoxalase' by Hopkins & Morgan (1948) is undoubtedly responsible for catalysing the formation of the intermediate compound and appears to be of low specificity so far as

the type of glyoxal is concerned. In order to explain the high specificity of glutathione as a 'co-enzyme', the specificity of 'glyoxalase' for the SH compound must, however, be high. Spontaneous breakdown of the intermediate accounts for the glyoxalase activity of the pure enzyme.

The second catalyst, which is here shown to be identical with the 'factor' of Hopkins & Morgan (1948), strongly catalyses the breakdown of the intermediate formed from methylglyoxal, thus accelerating the overall reaction. The specificity of this second enzyme appears to be higher than that of the first since its accelerating effect upon the intermediate formed from phenylglyoxal is shown to be low.

In heart tissue the activity of 'enzyme' appears to be greater than that of 'factor' when measured by the relatively greater rate at which SH groups disappear in the presence of methylglyoxal in comparison with their rate of reappearance. This suggests that in this tissue the rate-limiting step is the factor-accelerated breakdown of the intermediate. The spontaneous breakdown of the intermediate is certainly the rate-limiting step in the absence of factor. In the absence of factor the amount of 'enzyme' can be reduced much below that usually used without affecting the overall velocity. In heart extracts, too, the factor concentration is sufficiently low not to affect the velocity of breakdown of the phenylglyoxal intermediate. However, an investigation of the glyoxalase system in liver (unpublished experiments) indicates that in this tissue the enzymes are either more reactive or present in greater concentration than in heart. With factor preparations from liver it can be shown that the specificity of factor for the methylglyoxal intermediate is not absolute since high factor concentrations also catalyse the breakdown of the phenylglyoxal intermediate to some degree.

These experiments give little evidence for the type of intermediate compound involved or for the step at which the water molecule is incorporated. However, we would agree with Racker (1951) that an ester of thiolactic acid which could hydrolyse to lactic acid and GSH seems most likely to account for the observed properties, e.g.

$$\begin{array}{l} CH_3 \\ | \\ CHOH \\ | \\ CO(SG) + HOH \end{array} \rightarrow \begin{array}{l} CH_3 \\ | \\ CHOH \\ | \\ COOH + (GSH) \end{array}$$

The route suggested by Racker for the formation of this ester is, however, capable of numerous variations according to the order in which one postulates the various keto-enol tautomerizations, whether one assumes that GSH adds across C=C or C=O, and whether one invokes the hydrated or the anhydrous forms of the glyoxals. The reason adduced by Racker for preferring the anhydrous to the hydrated form of the glyoxal for the initial condensation with GSH is not valid. He suggests that the ensuing removal of water to form a C=C double bond, necessary if the hydrated glyoxal is the reacting species, would imply easy reversibility of the glyoxalase reaction as a whole since it is then analogous to easily reversible systems such as enolase and fumarase. While agreeing that no sign of reversibility can be detected in the glyoxalase system, we do not agree that this necessarily eliminates an enolase type of water removal since the irreversibility may well lie in the earlier glyoxal-glutathione condensation. It thus seems too early to make any decisions in favour of one particular set of intermediates.

There are no data bearing on the relationship of the 'biological' and 'chemical' compounds of the glyoxals and glutathione. It is possible that the 'chemical' compound is an intermediate in the formation of the 'biological' compound, although its low reactivity towards factor-free enzyme in the absence of excess methyl or phenylglyoxal might argue against this hypothesis. From Racker's (1951) results it would appear that he has found the methylglyoxal-GSH compound to be rather more stable than it has proved in our hands. However, we have worked at a somewhat higher pH (7·3–7·4 instead of 6·6) and, in general, our time scale has been longer. In addition, determinations of the rate of breakdown were done in the presence of excess methylglyoxal, which has been shown to accelerate this reaction. Apart from such small points the two sets of results, which were obtained quite independently with enzymes from different sources and using different methods of estimation, show a most satisfactory measure of agreement.

SUMMARY

1. The enzyme glyoxalase has been shown to consist of two parts. The first of these, identical with the glyoxalase enzyme of Hopkins & Morgan (1948), catalyses the formation of an intermediate compound between methyl- or phenylglyoxal and reduced glutathione. This is the biological compound of Yamazoye (1936), and under physiological conditions of temperature and pH, slowly breaks down to form glutathione and the hydroxy acid corresponding to the glyoxal. The enzyme alone is therefore able slowly to convert glyoxals to the corresponding hydroxy acids.

2. The second part of glyoxalase, identical with the glyoxalase factor of Hopkins & Morgan (1948), catalyses the breakdown of the intermediate formed from glutathione and methylglyoxal but not the intermediate formed from phenylglyoxal.

3. The compound of phenylglyoxal is preferentially formed when both methyl- and phenyl-

glyoxals are present together, and therefore phenylglyoxal prevents the factor from accelerating the conversion of methylglyoxal to lactic acid as was shown by Hopkins & Morgan (1948).

4. The presence of uncombined methyl- or phenylglyoxal accelerates the breakdown of the intermediate compounds.

The authors wish to thank Dr M. Dixon and Mr E. J. Morgan for details of the purification method for glyoxalase and for gifts of 'Yamazoye' intermediate compounds.

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Studies in Carotenogenesis

5. CAROTENE PRODUCTION BY VARIOUS MUTANTS OF *PHYCOMYCES BLAKESLEEANUS* AND BY *PHYCOMYCES NITENS*

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The general conditions governing carotene synthesis in *Phycomyces blakesleeanus* have been worked out in detail (Garton, Goodwin & Lijinsky, 1951) and the polyenes which accompany β-carotene (the major component) in traces have also been identified (Goodwin, 1952). As a number of morphological mutants of *P. blakesleeanus* have been isolated and were available, it was considered desirable to examine them quantitatively and qualitatively for carotene synthesis. It was thought possible that some metabolic block might have occurred which would help in the elucidation of the pathway of biosynthesis of carotene. The mutants examined were *P. blakesleeanus* var. *piloboloides* (+ and − strains), *P. blakesleeanus* mutant *gracilis* (+ strain only), *P. blakesleeanus* mutant *pallens* (− strain). *P. nitens* (+) was also examined.

A short account of this work has already appeared (Goodwin & Griffiths, 1952).

EXPERIMENTAL

Cultures. All the cultures used were obtained from the Centraalbureau voor Schimmelcultures, Baarn, Holland.

Cultural methods. The fungi were grown and analysed for dry weight, lipid and carotene production as described in detail by Garton *et al.* (1951). The standard medium containing 2·5% (w/v) glucose was used throughout.

Identification of the polyenes present. The methods described by Goodwin (1952) were used.

RESULTS

The polyenes produced. A chromatographic examination of the polyenes present in the various mutants was carried out. All the mutants contained the same polyenes as are found in the parent strain, namely: lycopene, neurosporene, α-, β-, γ- and δ-carotene, phytofluene and a phytoene-like substance. These were also present in essentially the same relative amounts as in the parent form of *P. blakesleeanus*.

Production of carotene. Typical results obtained with the various strains are recorded in Table 1 and Figs. 1 and 2. In the first place it will be seen from Fig. 1 that there are no significant differences between the three (−) strains in either the rate of synthesis or the amount of carotene finally produced. There is also no major difference between the amount of dry weight and lipid synthesized by these strains (Table 1).

When the (+) strains are considered (Fig. 2), *piloboloides* and *nitens* fall into line with the (+) strain of the parent in synthesizing about half as much carotene as the (−) strain (Garton *et al.* 1951), but the same amount of dry weight and lipid (Table 1). The mutant *gracilis* (+), however, is different in two respects: (*a*) it synthesizes as much carotene as the (−) strains of the other variants, and (*b*) the rate of synthesis in young cultures is very

Table 1. *The dry weight, lipid and carotene production by various strains of* Phycomyces blakesleeanus *and by* Phycomyces nitens *when grown on a standard medium containing* 2·5 % (*w*/*v*) *glucose*

(The amounts recorded are those produced in an 8 oz. medicine bottle containing 15 ml. of medium.)

Days after inoculation ...	3				4				5			
Strain used	Dry wt. (mg.)	Lipid (mg.)	Lipid (%)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Lipid (%)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Lipid (%)	Carotene (μg.)
P. blakesleeanus (−)	42	7·3	17·4	11	77	19·3	25·1	26	74	20·3	27·3	43
P. blakesleeanus var. *pilobolus* (+)	40	7·2	18·0	4	70	13	18·6	20	80	18·5	23·2	85
P. blakesleeanus var. *pilobolus* (−)	52	9·7	18·7	9	66	14·8	22·4	22	78	20·4	26·2	43
P. blakesleeanus mutant *gracilis* (+)	59	15·0	25·4	36	—	—	—	—	65	16·6	25·5	79
P. blakesleeanus mutant *pallens* (−)	36	6·9	19·1	9	66	8·4	12·7	16	79	12·9	16·3	44
P. nitens (+)	57	15·8	27·8	20	—	—	—	—	65	15·7	24·1	29

Days after inoculation ...	7				9				11			
Strain used	Dry wt. (mg.)	Lipid (mg.)	Lipid (%)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Lipid (%)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Lipid (%)	Carotene (μg.)
P. blakesleeanus (−)	96	16·1	16·8	92	79	15·7	19·9	97	85	14·0	16·5	100
P. blakesleeanus var. *pilobolus* (+)	79	15·1	19·1	30	86	20·3	23·6	35	77	12·0	15·6	35
P. blakesleeanus var. *pilobolus* (−)	—	—	—	—	96	24·8	25·8	97	81	17·0	21·0	95
P. blaksleeanus mutant *gracilis* (+)	80	17·5	21·9	99	80	18·1	22·6	108	—	—	—	—
P. blakesleeanus mutant *pallens* (−)	102	23·7	23·2	115	97	28·2	10·9	109	—	—	—	—
P. nitens (+)	67	12·5	18·6	31	62	10·5	16·9	24	—	—	—	—

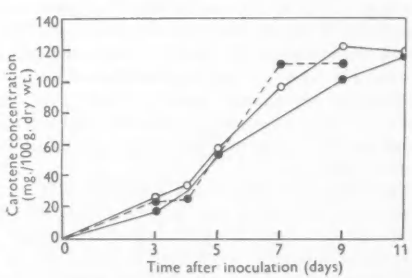

Fig. 1. The concentration of β-carotene at various stages of growth in various strains of *Phycomyces blakesleeanus*. *P. blakesleeanus* (−), ○—○; *P. blakesleeanus* var. *piloboloides* (−), ●- - -●; *P. blakesleeanus* mutant *pallens* (−), ●—●.

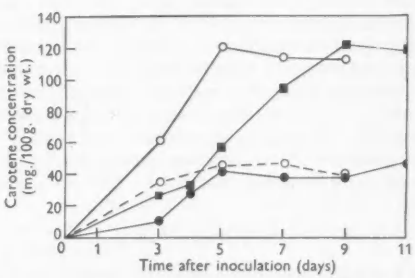

Fig. 2. The concentration of β-carotene at various stages of growth in various strains of *Phycomyces blakesleeanus* and in *Phycomyces nitens* (+). (The curve for the parent strain is repeated in this figure for ease of comparison.) *P. blakesleeanus* (−), ■—■; *P. blakesleeanus* var. *piloboloides* (+), ○- - -○; *P. blakesleeanus* mutant *gracilis* (+), ○—○; *P. nitens* (+), ●—●.

much greater than in every other strain examined (Fig. 2). Lipid synthesis was essentially the same in all the (+) strains and did not differ from that in the (−) strains. The same conclusions apply to dry-weight productions except in the case of *P. nitens*. The normal dry-weight production by this strain in an 8 oz. medicine bottle is about 65 mg., whilst all the other strains produce between 80 and 100 mg.

DISCUSSION

All the morphological mutants of *P. blakesleeanus* examined, with the exception of *P. blakesleeanus* mutant *gracilis* (+), produce the same polyenes in the same amounts as do the (+) and (−) strains of the parent. From the point of view of helping to provide insight into the intermediary steps in the biogenesis of polyenes (in particular β-carotene), they must be considered as of no particular value. The mutant *gracilis* (+), however, falls out of line in two respects: it synthesizes as much carotene as other (−) strains (i.e. about twice as much as the normal (+) strains) and it does so at a very much greater rate. There is, however, no difference in the type of polyenes synthesized or in their relative amounts.

Carotene production in the parent strain is comparatively small during the growth of the mycelium, the greater part being synthesized after growth is complete (Goodwin & Willmer, 1952). As an explanation of this it has been suggested that carotene is synthesized from the products of the anaerobic dissimilation of glucose, the opportunities for this type of metabolism being greatest when the mycelium is completely formed as a mat covering the surface of a static culture; it is also possible that the demand for carotene precursors is only fully satisfied when the fungus is fully grown. Whatever the final explanation, it is clear from Fig. 2 that in *gracilis* (+) the situation is different. There are not two rates of carotenogenesis according to whether the fungus is growing or not. In this strain the rate of synthesis is almost exactly the same as the rate of growth. When more is known about the details of carotene synthesis, it will obviously be of interest to re-examine *gracilis* (+), but at the present state of knowledge, it is not likely to be of any more value than the parent. The (+) and (−) strains of the Phycomycetes are considered to be the two sexual forms. Apart from their fundamental property of conjugating to form zygospores, the two forms are often characterized by their ability to synthesize different amounts of carotene (see Goodwin, 1950). Whether this is related to their sexual reactions or is purely fortuitious is not known. The present series of experiments does little to provide an answer to this problem. All the strains examined, except *gracilis* (+), conform to the general observation that the (−) strains produce about twice as much carotene as do the (+) strains. As previously stated, in this connexion *gracilis* (+) behaves as a (−) strain. It is observations of this type which make it doubtful that carotene production has any direct function in the reproduction of the Phycomycetes. In this connexion it would have been very interesting to have examined *gracilis* (−), but apparently this strain has not yet been isolated.

SUMMARY

1. The growth, lipid and carotene production of the following have been compared with those of *Phycomyces blakesleeanus* (−): *P. blakesleeanus* var. *piloboloides* (+ and − strains), *P. blakesleeanus* mutant *gracilis* (+), *P. blakesleeanus* mutant *pallens* (−) and *P. nitens* (+).

2. Lipid production was the same in all; growth was the same in all but *P. nitens*, which produced about 70% of the dry weight obtained with the others.

3. Carotene synthesis in all the (−) strains was the same; in all the (+) strains except *gracilis* (+), carotene synthesis was about one-half of that in the (−) strain. The *gracilis* (+) mutant produced as much as a normal (−) strain.

4. The rate of synthesis of carotene was the same in all except *gracilis* (+), in which it was much more rapid.

5. The same polyenes occurred in all the mutants and in the same relative amounts.

We wish to thank Prof. R. A. Morton, F.R.S., for his continued interest in this work, which was carried out during the tenure (by L. A. G.) of a University of Liverpool Fellowship. Thanks are also due to the Medical Research Council for a grant towards laboratory expenses.

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Potentiometric Determination of Chloride in Biological Fluids

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Potentiometric titration of chloride with silver nitrate has been a standard procedure for many years, and the theoretical considerations involved have been clearly set forth by Kolthoff & Furman (1931). However, methods based on this principle have been little used in biological work, in spite of their attractive possibilities. Lehmann (1939) seems to have been the first to describe a method suitable for clinical work; more recently, Norberg (1949) has proposed a similar procedure, differing from Lehmann's mainly in the arrangements for measuring potential difference. Neither of these methods exploits to the full the great convenience and speed which is possible with potentiometric titrations; the method here described is an adaptation of Lehmann's, but is considerably quicker and more convenient. Chloride can be determined in 0·2 ml. of plasma or urine, and when plasma is being analysed removal of protein is unnecessary.

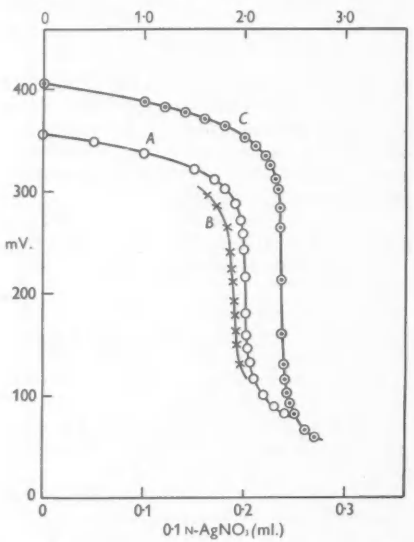

Fig. 1. Titration curves of 0·2 ml. 0·1 N-NaCl (*A*); 0·2 ml. normal human plasma (*B*); and 1 ml. normal human urine (*C*). Abscissae (ml. of 0·1 N-$AgNO_3$) for (*A*) and (*B*) at lower border of figure and for (*C*) at upper border.

When silver nitrate is added to a solution containing chloride ions, the concentration of silver ions in the solution is at first very low. As more silver nitrate is added, the silver-ion concentration rises slowly, and then more rapidly as the equivalence point is reached. When this point is passed, and all the chloride is precipitated as silver chloride, silver ions accumulate rapidly.

These changes can be followed potentiometrically, by immersing a silver electrode and an indifferent electrode in the solution and measuring the potential difference between them. If this p.d. is plotted against the volume of silver nitrate added, a sigmoid curve, like that illustrated in Fig. 1, *A*, is obtained. It has been shown (Kolthoff & Furman, 1931) that the equivalence point either coincides with the centre of the steepest portion of the curve, or is separated from it by a negligible distance.

EXPERIMENTAL

Apparatus

The apparatus consists of a microburette (0·25 ml. or 2 ml.), a stirring device, indifferent and silver electrodes, and an instrument to indicate the potential difference. The latter must present a high resistance to the electrodes, since the passage of a current through the cell causes electrolysis, which may vitiate the results unless the amount is kept very small. In the arrangement described by Lehmann (1939), the instrument used was a slide-wire potentiometer of the usual null-point type, requiring the adjustment of a dial for every reading. In the present arrangement this has been replaced by an electronic millivoltmeter which has a moderately high input resistance (5 MΩ) and at the same time provides continuous indication of the input voltage on a robust moving-coil type of instrument. This greatly simplifies the procedure of titration and gives visual indication of the approach of the end point. This instrument is described in the Appendix. If a direct reading electronic millivoltmeter is already available, e.g. in the form of a pH meter, it will serve the purpose admirably; the pH meter type of instrument, however, has a much higher input impedance than is necessary for the present purpose and is likely to be more costly in consequence.

The silver electrode, as in Lehmann's method, is simply a piece of 20 s.w.g. silver wire dipping into the solution, but for the indifferent electrode another modification has been introduced. Lehmann used a calomel or quinhydrone electrode, connected to the titration vessel by a potassium nitrate agar bridge. Such bridges are inconvenient, being somewhat troublesome to prepare and maintain; when not in use, the free end must be kept immersed in saturated

potassium nitrate solution, as, if exposed to the air, the agar shrinks, leaving a small 'dead space' at the end of the tube which readily traps a bubble of air and thus breaks the continuity of the circuit. By an ingenious device due to Willard & Boldyreff (1929) the use of bridges may be avoided and one of the elements dipping into the titration vessel eliminated. A piece of platinum wire is sealed into the wall of the burette below the tap, and the outer end of this connected by a wire to the voltmeter. The platinum is thus immersed in a solution of constant composition, and takes up a constant potential with respect to it. The circuit is completed by dipping the burette tip (which should be of fine bore, to minimize diffusion) under the surface of the titration mixture.

Some means of stirring the mixture must be provided. The author uses the stream of air from a vibrating-reed pump of the type used to aerate the water of aquaria. A mechanical shaking device, or a magnetic stirrer could no doubt be used instead.

The type of burette used depends on the size of sample being analysed. If plasma, or small amounts of urine, are being analysed, 0·2 ml. is taken and a Conway burette (capacity 0·25 ml.) has proved very convenient. If larger amounts of urine are available a sample of 1 ml. can be taken and this will give a sharper end point; a larger burette is then required and the author uses a 2 ml. burette graduated in divisions of 0·02 ml. In both cases the burette requires the minor modification of a platinum wire sealed in below the tap.

Reagents

Silver nitrate (0·1 N); acetic acid 50% (v/v); octan-2-ol ('capryl alcohol').

Procedure

In the classical method of potentiometric titration, a graph is prepared of every titration and the end point determined by inspection. In this method, however, the conditions are so constant that this is unnecessary; the end point occurs at the same potential with every titration, whether plasma, urine or standard NaCl solution is being titrated. With the author's apparatus, this potential lies between 190 and 200 mV. $AgNO_3$ is added until this potential is shown by the meter which is used exactly like an indicator in an ordinary titration. Protein does not alter the end-point potential: in its presence, the curve is not quite so steep, but the centre of the steep portion remains at the same potential (Fig. 1, *B*).

Although the end-point potential with the author's instrument has proved quite constant, this potential should be determined independently for other instruments by constructing titration curves in the usual way.

For plasma, the procedure is as follows: acetic acid (5 ml. of 50% v/v) is pipetted into a flat-bottomed glass tube (2 × 7·5 cm.). Plasma (0·2 ml.) is added from a washout pipette, together with 1 or 2 drops of octan-2-ol (to prevent foaming). The meter is adjusted for sensitivity and zero, and the glass tube is placed in position under the burette (Fig. 2). A potential of the order of 350 mV. should now be registered on the meter. $AgNO_3$ (0·1 N) is next added from the burette: at first there is little change in potential and the solution can be run in rapidly, but as the end point is approached the potential begins to change more quickly and when it has reached 300 mV. the $AgNO_3$ should be added more cautiously.

With the Conway burette volumes of 0·001 ml. can be added accurately and as Fig. 1 shows, there is no difficulty in deciding on the end point with additions of this order. A short time is required for the potential to change after each addition of silver, and at or near the end point it is necessary to wait for some 10–15 sec. before reading the meter, otherwise the end point will be overshot. For this reason, the 'magic eye' type of indicator is unlikely to be satisfactory, as it gives insufficient warning of the approach of the end point. If x ml. of 0·1 N-$AgNO_3$ nitrate are used in the titration, then the sample contains $100x/0·2 = 500x$ m-equiv. of chloride per l.

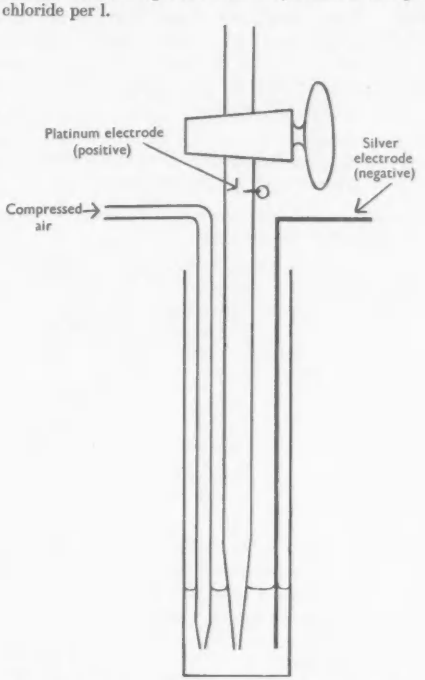

Fig. 2. Arrangement of apparatus for titration. (Scale one-half actual size.)

The procedure for urine is very similar. If only small amounts are available, a sample of 0·2 ml. can be analysed as described above with entirely satisfactory results. However, if larger amounts are available, 1 ml. samples give a sharper end point (Fig. 1, *C*) and are more convenient to pipette. Acetic acid (5 ml.) is used as before and the mixture is titrated with 0·1 N-$AgNO_3$ from a 2 ml. burette, until the meter reads 190–200 mV. If x ml. of 0·1 N-$AgNO_3$ are required for titration, then the sample contains $100x$ m-equiv. of chloride per l.

RESULTS

Accuracy

The accuracy of the method was tested in two separate ways: by recovery experiments after the addition of known amounts of chloride to plasma

and urine, and by parallel determinations of chloride in plasma and urine by the present method and by Van Slyke & Hiller's (1947) modification of Sendroy's (1937) iodometric method.

Recovery experiments

Plasma. A number of samples of human plasma were pooled and dialysed in a cellophan sac against distilled water for 24 hr., the water being changed after the first 12 hr. At the end of this time the plasma contained less than 0·5 m-equiv./l. of chloride. Known amounts of sodium chloride were then added to samples of this dialysed plasma, and the samples titrated by the usual procedure. The results are shown in Table 1.

Table 1. *Recovery of chloride added to dialysed plasma and to urine low in chloride*

(Results of chloride recovered expressed to nearest 0·5 m-equiv./l.)

Chloride added (m-equiv./l.)	Chloride recovered (m-equiv./l.)	Error (%)
	Dialysed plasma	
80	80	0
100	99·5	−0·5
120	119	−0·8
	Urine	
50	50	0
100	101·5	+1·5
150	150·5	+0·3
200	201	+0·5
250	250	0

Urine. A similar procedure was used; urine low in chloride was obtained from a case of cardiac failure, and known amounts of sodium chloride were added. The results are shown in Table 1.

Parallel determinations

The results of chloride estimations of four samples of plasma and five samples of urine by the present method and by the method of Van Slyke & Hiller (1947) are shown in Table 2. The plasma samples were obtained from a normal subject in the course of an infusion of 10 % (w/v) sodium chloride solution, which accounts for the high chloride figures.

In the course of preliminary experiments with Van Slyke & Hiller's method, it was found that the results obtained with plasma, when tungstic acid was used to precipitate proteins, were consistently 1–2 m-equiv./l. higher than those obtained by the electrometric method. When, however, picric acid was used instead of tungstic acid, good agreement between the two methods was found. In the plasma determinations by the method of Van Slyke & Hiller quoted in Table 2, picric acid was used.

Table 2. *Parallel determinations of chloride in plasma and urine by electrometric titration and by the method of Van Slyke & Hiller* (1947)

		Chloride content (m-equiv./l.)	
		Electrometric method	Van Slyke & Hiller method
Plasma	1	104·5, 104·5	104·8, 105
	2	124·6, 125·2	125, 125
	3	123·6, 122·6	122·8, 123·2
	4	113·8, 113·8	113·7, 114
Urine	1	220·2, 222·2	220·2, 220·5
	2	273, 273	271, 271·8
	3	13·8, 13·8	12·6
	4	137, 137·5	137·3, 137·5
	5	118·7, 118·7	117·8, 118·1

Van Slyke & Hiller noted this difference, but were unable to account for it; they showed that contamination of the sodium tungstate with chloride was not the cause. They also found that a reference plasma chloride method (Van Slyke, 1923–4) gave results agreeing better with the picric acid procedure than with the tungstate method. The results reported here suggest that when the method of Van Slyke & Hiller is being used for plasma, and when results of the highest accuracy are required, picric acid should be used for the removal of proteins in preference to tungstic acid.

Interfering substances

The presence of phosphate or sulphate in concentrations up to 100 mM has no effect on the results. Oxalate can be used as anti-coagulant without introducing any error apart from that due to red-cell shrinkage.

Bromide and iodide both interfere, giving results which are too high by the amount of extra halide present. Thiosulphate also interferes since the solubility of silver thiosulphate upsets the relation between silver-ion concentration and amount of chloride present.

SUMMARY

A rapid and accurate method of determining chloride in small amounts (0·2 ml.) of biological fluids is described. The method depends upon the electrometric titration of the chloride in the sample with silver nitrate, using a directly indicating electronic millivoltmeter.

My attention was drawn to the possibility of an electrometric method for chloride in plasma by Dr J. N. Hunt. Some of the earlier development of the method was done in collaboration with Dr A. H. James. I am grateful to Mr P. W. Roberts for making me familiar with the virtues of the cathode-follower as an impedance transformer, and to Messrs Ionic Instruments Ltd. for certain improvements on the original circuit.

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APPENDIX

Circuit of millivoltmeter

The circuit of the electronic millivoltmeter is shown in Fig. 3. The input voltage is applied across the grids of a double triode valve connected as a double cathode-follower. The output voltage appears across the cathodes and is measured by a moving coil meter in series with an adjustable resistance. The input impedance is 5 MΩ, but because of the low output impedance of a cathode-follower circuit, appreciable current (50 μA.) can be drawn by the meter without interfering with the input circuit. After an initial 'warming-up' period of 10 min. or so, the instrument exhibits very great inherent freedom from 'drift', and the zero and sensitivity remain unchanged when the mains voltage is varied between 180 and 250. R_{11} is the zero adjustment, and R_9 the sensitivity control. Calibration is carried out as follows. S_1 is operated so that the contacts represented in Fig. 3 as being lowermost are made. (The meter is now connected directly, via R_5, to the voltage dividing chain across B.) R_2 is adjusted until the meter reads 500 mV. S_1 is now turned to its middle position; this applies an input of 500 mV. to the valve, and transfers the meter to its normal working position across the cathodes. R_9 is now adjusted until the meter again reads 500 mV. S_1 is finally turned to its 'uppermost' position and the instrument is ready for use. R_2 and R_9 should require only occasional adjustment.

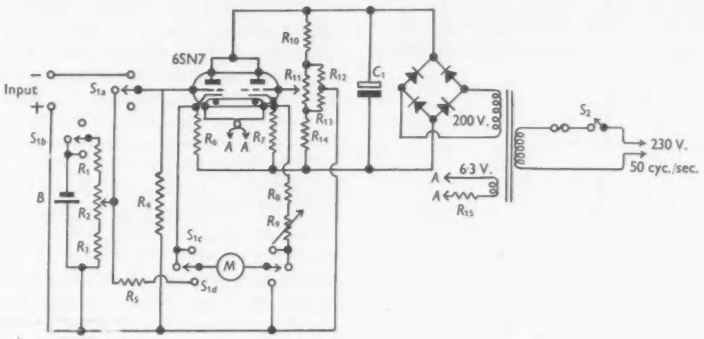

Fig. 3. Circuit of amplifier (for references see list of components).

Component values are as follows: R_1, 60 Ω; R_2, 20 Ω; R_3, 30 Ω; R_4, 5 MΩ; R_5, 10 000 Ω minus meter resistance; R_6, R_7, 500 000 Ω; R_8, 4000 Ω; R_9, 2500 Ω; R_{10}, 20 000 Ω; R_{11}, 500 Ω; R_{12}, R_{13}, 200 Ω; R_{14}, 75 000 Ω; R_{15}, 2·5 Ω; C_1, 16 μF. electrolytic, 350 V. working; S_1, 4-pole, 3-way wafer switch, Yaxley type; S_2, on-off toggle switch; B, 1·5 V. dry battery, size U2; M, 0–50 microammeter, 3·5 in. scale, marked 0–500 mV. (Pullin).

All resistors and potentiometers should be wirewound, except R_4, R_6 and R_7, which should be of the 'cracked-carbon' type.

An instrument constructed to the above specification can be obtained from Ionic Instruments Ltd., 14 D Princes Mews, London, W. 2.

The Alleged Effect of Acid Phosphate on the Anion Permeability of Erythrocytes

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Keilin & Mann (1941) described an optical method of studying the penetration of erythrocytes by the bicarbonate ion and were able to demonstrate in an elegant manner the activity of carbonic anhydrase within the cells and the link between this activity and Hamburger's chloride shift. Their method consists in converting the haemoglobin inside the cell to methaemoglobin and then observing the transformation of the methaemoglobin from the acid to the alkaline form by the entering bicarbonate. In their experiments sodium carbonate solution is added to a suspension of methaemoglobin cells in

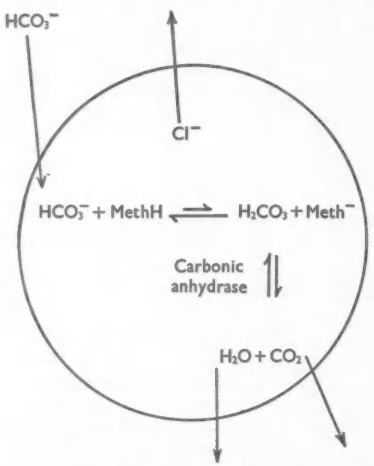

Fig. 1. The changes that occur when Na_2CO_3 solution is added to a suspension of methaemoglobin cells. The loss of water is accompanied by cell shrinkage.

sodium chloride solution and the following changes occur: (1) external bicarbonate exchanges rapidly for internal chloride, a process completed within 2 sec. (Dirkin & Mook, 1931; Luckner & Lo-Sing, 1938); (2) acid methaemoglobin reacts with bicarbonate forming alkaline methaemoglobin and carbonic acid; (3) carbonic acid is dehydrated, a reaction catalysed by intracellular carbonic anhydrase; (4) carbon dioxide and water leave the cell, and it shrinks (Warburg, 1922; Ponder, 1948). These changes are illustrated in Fig. 1.

Keilin & Mann (1941) and Keilin & Hartree (1946) studied the change of acid to alkaline methaemoglobin spectroscopically and found it to be practically complete after 90 sec. They also noticed that cells washed previously in acid phosphate or in phosphate, phthalate or citrate buffers of pH 6 do not change colour in this way. This phenomenon, which is the subject of the present paper, will be referred to as the Keilin & Mann effect. These workers suggested that such washing destroys the permeability of the cells to anions. A second possible explanation of the effect is that the cell has lost its rapidly diffusing chloride ions in exchange for slowly moving phosphate, phthalate or citrate ions and is thus unable to accept incoming bicarbonate.

These two hypotheses have been tested, in so far as they apply to phosphate, by spectroscopic observations, measurement of cell shrinkage and chemical analyses, using conditions in which the cells developed the Keilin & Mann effect gradually. The link between the change of methaemoglobin colour and cell shrinkage was confirmed, and the Keilin & Mann effect studied by photometric measurement of the shrinkage. Cells were treated with phosphate buffer and examined at different times for (*a*) the Keilin & Mann effect, (*b*) the phosphate content, and (*c*) the chloride exuded. A consistent relationship was found between these quantities, while no changes in cell permeability were observed.

METHODS

Solutions. These had the following compositions. Saline, 0·154 M-NaCl; $NaNO_2$, 0·154 M; Na_2CO_3, 0·2 M; phosphate buffer, pH 6, 87·5 ml. 0·2 M-NaH_2PO_4 + 12·5 ml. 0·2 M-Na_2HPO_4; glucose, 0·296 M.

Preparation of methaemoglobin cells. Defibrinated horse blood was stored at 5° and used 1–2 days after collection. The separated erythrocytes were washed three times with saline, once with $NaNO_2$ solution and four times with saline. The volume of washing fluid was always twice the volume of the centrifuged cells. The washed cells were finally mixed with an equal volume of saline to give a concentrated suspension.

Photometric study of cell shrinkage. 1 drop of the concentrated suspension was mixed with saline in the cell of a King colorimeter using full aperture and no filter. The mixture was diluted gradually with saline until the instrument gave a reading of +3 scale divisions. The volume was adjusted to 5 ml. by withdrawing the excess, 5 drops of Na_2CO_3 solution

were added, a stop-watch started, the suspension mixed well and readings of the photometer deflexion taken at 5 or 10 sec. intervals until they became steady. This occurred after 90–120 sec. The total deflexion of between +10 and +25 scale divisions (depending on the previous treatment of the cells) was reproducible to within ±1 scale division. The experiment was repeated once or twice and the mean total deflexion noted. The technique is similar to that used by Parpart, Jacobs & Dzienmian (1937) in following chloride-sulphate exchanges of erythrocytes.

The initial adjustment of the concentration of the cell suspension until it gave a reading of +3 in the photometer was adopted as a convenient method of preparing a standard suspension. A subsidiary experiment showed that the further dilution caused by adding 5 drops of saline to 5 ml. of such a suspension brought the photometer reading from +3 to 0. Thus the method also allows for the dilution caused by the addition of 5 drops of Na_2CO_3 solution.

The total galvanometer deflexion obtained in such an experiment is dependent on both the shrinkage of the cells and their colour change. The contribution of each phenomenon to the total effect was determined as follows.

Direct counting showed that the standard suspension contained $9{\cdot}7 \times 10^4$ cells/μl. For a suspension of this concentration in a 1 cm. cell, Drabkin & Singer (1939) have shown that

$$E_T = E_S + E_P,$$

where E_T is the total extinction, E_S is the scattering extinction, and E_P is the extinction of the haemolysed suspension, i.e. that due to the total pigment of the cells when free in solution. Thus, the total change of extinction upon adding Na_2CO_3 is given by the expression

$$\Delta E_T = \Delta E_S + \Delta E_P$$

and the resulting change of colorimeter reading Δx_T is given by the expression

$$\Delta x_T = \Delta x_S + \Delta x_P.$$

Δx_P was measured indirectly by haemolysing a suspension three times as concentrated as a standard suspension and noting the change of colorimeter reading on the addition of Na_2CO_3 to the haemolysate. Allowing for the dilution effect as before, the value of Δx_P for a standard suspension was calculated to be $+2{\cdot}6 \pm 0{\cdot}2$ scale divisions. The total deflexion Δx_T of untreated cells was 20–25 scale divisions. About 10% of the total deflexion was thus due to the pigment colour change and about 90% to cell shrinkage.

Analysis of cell suspensions for phosphate. A sample (2 ml.) of the well shaken suspension was haemolysed by adding 5 ml. of water and 0·1 ml. of saponin solution, 2 ml. of 25% (w/v) trichloroacetic acid were added, the volume made up to 10 ml. and the whole stirred and filtered. Between 0·5 and 7 ml. of the clear filtrate were analysed for inorganic phosphate by the method of Lohmann & Jendrassik (1926).

The concentration of cells in the suspension was determined by adding 0·5 ml. to a haematocrit tube and reading the volume of packed cells after centrifuging for 15 min. at 1500 *g*. The phosphate content was calculated in terms of μmoles of P/ml. of packed cells.

Subsidiary experiments in which the volume of a sample of cells was determined before and after treatment with phosphate buffer followed by saline washing as described above showed that no net change in volume was produced by this treatment.

Analysis of chloride exuded from the cells. Washed, centrifuged cells were mixed with about seven times their volume of phosphate buffer. At intervals 15 ml. of the well shaken suspension were withdrawn and centrifuged, and two 5 ml. samples of the supernatant analysed by Volhard titration following the procedures of Peters & Van Slyke (1932) for urine.

RESULTS

The shrinkage of cells upon the addition of carbonate

When 5 drops of sodium carbonate solution were added to a dilute suspension of methaemoglobin cells untreated with acid buffers they gave a gradually increasing photometer deflexion reaching a final value of 20–25 scale divisions depending upon the blood sample. The time course of the deflexion, which mainly represents cell shrinkage, followed the curve shown in Fig. 2, curve *A*. It is practically complete after 90 sec. and quite complete after 120 sec.

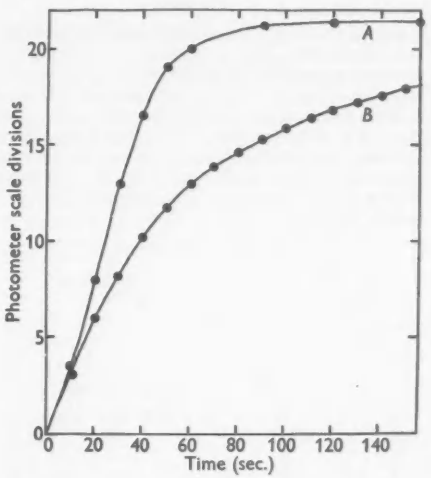

Fig. 2. The time curve of cell shrinkage when Na_2CO_3 solution is added to a dilute suspension of methaemoglobin cells in a colorimeter cell. Curve *A*, cells suspended in saline. Curve *B*, cells suspended in saline containing 5×10^{-4} M-sulphanilamide.

The shrinkage and colour change are linked with the reaction catalysed by carbonic anhydrase, as illustrated in Fig. 1. This was confirmed by the effect of treating the cells with sulphanilamide, which is a specific inhibitor of this enzyme (Keilin & Mann, 1940), before adding the sodium carbonate. In the presence of 5×10^{-4} M-sulphanilamide the photometer deflexion was greatly slowed (Fig. 2, curve *B*).

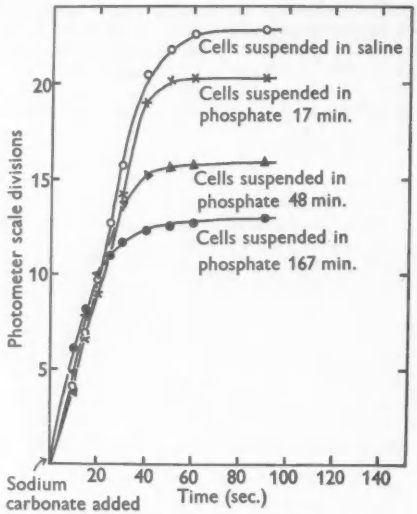

Fig. 3. The effect of pre-treatment with acid phosphate upon the shrinkage of methaemoglobin cells when sodium carbonate is added to a dilute suspension. A concentrated suspension of washed methaemoglobin cells was mixed with an equal volume of phosphate buffer, pH 6. After standing at room temperature (20°) for a measured time the cells were centrifuged, washed twice with saline and resuspended in twice their volume of saline. The shrinkage of these cells on the addition of carbonate was studied as described in the text.

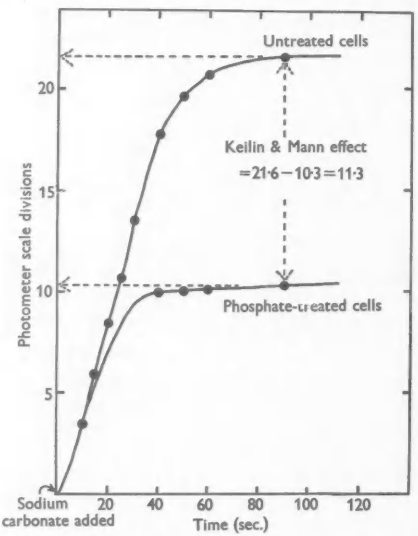

Fig. 4. The quantitative measure of the Keilin & Mann effect.

The effect of acid phosphate buffer on cell shrinkage and on the colour change of methaemoglobin

Methaemoglobin cells treated with phosphate buffer showed a reduced photometer deflexion on the addition of sodium carbonate, depending upon the period of treatment with phosphate (Fig. 3). The velocity of the shrinkage was unaffected, but its extent was reduced by such treatment. These cells also exhibited only a partial change in absorption spectrum when studied in the microspectroscope of Keilin & Mann (1941). When the total deflexion resulting from sodium carbonate addition had been reduced to about 10 scale divisions by sufficiently long treatment with phosphate no change in absorption spectrum was observed on adding carbonate. The reduction in photometer deflexion caused by treatment with phosphate buffer was taken as a quantitative measure of the Keilin & Mann effect (see Fig. 4). Prolonged treatment with phosphate failed to reduce the deflexion below 8 scale divisions.

The relation between the Keilin & Mann effect and the phosphate-chloride exchange

The phosphate content of cells treated for various periods with phosphate buffer is plotted in Fig. 5 against the Keilin & Mann effect shown by the same cells. In Fig. 6 the chloride content of the supernatant phosphate buffer obtained and analysed as described is plotted against the Keilin & Mann effect exhibited by cells separated from these supernatants. The Keilin & Mann effect is seen to rise steadily with the phosphate absorbed and also with the chloride exuded by the cells.

Reversibility of phosphate penetration

Keilin & Hartree (1946) found that the effect of acid phosphate treatment in preventing the entry of bicarbonate into the cells was irreversible. By following the phosphate content of cells for several hours, it has now been possible to show that this apparent irreversibility is due to the very slow rate at which phosphate leaves the cell. Methaemoglobin cells suspended in saline were found to be too fragile for such protracted experiments and normal erythrocytes were therefore studied.

The rate of penetration of phosphate into normal erythrocytes suspended in phosphate buffer pH 6 was first studied by direct chemical analysis. The results, given in Fig. 7, curve *A*, show that the phosphate content rises logarithmically with time, attaining equilibrium in 4–5 hr. The rate of loss of phosphate from erythrocytes that had been washed three times in phosphate and then suspended in saline pH 7·3 was also studied by direct analysis. Fig. 7, curve *B*, shows that the phosphate content falls logarithmically with time, but that the rate of exit is about one-tenth that of entry.

DISCUSSION

The unchanged permeability of cells washed with acid phosphate

The rate of cell shrinkage and colour change on the addition of carbonate depends on the overall rate of the process shown in Fig. 1. Any reduction of the rate of transfer of bicarbonate or chloride ions into and out of the cell will appear only if the rate of this ionic exchange becomes the limiting factor of the whole process. The results illustrated in Fig. 3 indicate that washing with acid phosphate buffer has no such effect. Thus it may be concluded that the permeability of the cells to these anions is not destroyed and is probably unaffected by such washing.

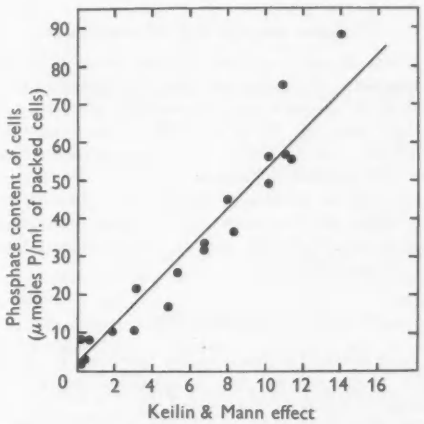

Fig. 5. The relation between the phosphate content of cells and their Keilin & Mann effect. The cells were washed free of intercellular phosphate before analysis.

The phosphate-chloride exchange

The results given in Figs. 5 and 6 show that an exchange of external phosphate for internal chloride occurs when cells are treated with acid phosphate buffer. As Booth (1938) has pointed out, if rapidly diffusing chloride ions inside the cell are replaced by slowly diffusing phosphate ions, the amount of bicarbonate ion which can then enter the cell rapidly is limited by the extent of the replacement. This has now been confirmed by the direct relation shown to exist between the extent of the phosphate-chloride exchange and the reduction in the cell shrinkage, which is itself linked with the entry of bicarbonate (Fig. 1).

It is clear that this replacement is responsible for the action of acid phosphate in decreasing and finally abolishing the acid methaemoglobin → alkaline methaemoglobin reaction since this reaction is also dependent upon the penetration of the cell by bicarbonate.

Keilin & Hartree (1946) found that citrate and phthalate buffers of pH 6 produced the same effect as acid phosphate. It remains to be shown whether these buffers act by the same mechanism as phosphate or whether some other mechanism is involved.

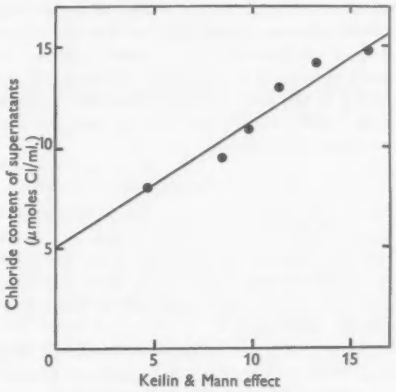

Fig. 6. The relation between the chloride content of the suspending fluid and the Keilin & Mann effect of the cells. Cells were transferred from saline to phosphate buffer. The rise in the chloride content of the phosphate buffer indicates a progressive loss of chloride from the cells. The cells were not washed free of intercellular chloride before suspension in phosphate. The intercept on the ordinate indicates the original intercellular chloride.

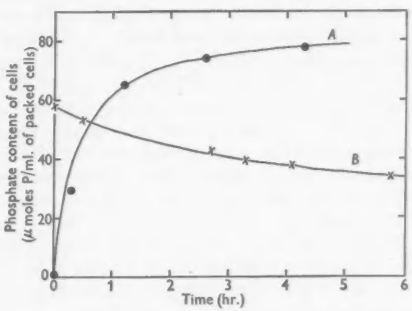

Fig. 7. Curve *A*, the time course of phosphate entry into washed horse erythrocytes suspended in phosphate buffer, pH 6·3, temp. 20°. Curve *B*, the time course of phosphate exit from phosphate-treated horse erythrocytes suspended in saline, pH 7·3, temp. 20°.

Mechanisms of cell shrinkage caused by carbonate

The shrinkage of saline-washed methaemoglobin cells upon the addition of carbonate to a saline suspension seems to be comprised of two parts.

That part which is studied in this paper is associated with the bicarbonate-chloride exchange, is abolished by previous treatment with acid phosphate, is slowed by sulphanilamide, and is accompanied by a change of internal pH. It is satisfactorily explained by the processes illustrated in Fig. 1. The other part of the shrinkage is unaffected by sulphanilamide, since in Fig. 2 the initial slopes of curves *A* and *B* are identical, is not abolished by prolonged treatment with acid phosphate, and does not change the colour of intracellular methaemoglobin. It is probably caused by the change in external pH, which according to the theoretical predictions of Jacobs & Stewart (1947) itself produces an osmotic loss of water and cell shrinkage.

The rates of phosphate entry and exit

There have been several demonstrations that phosphate enters the erythrocytes of different species (see Davson & Danielli, 1943, for review). Many of these have been only semi-quantitative and have used indirect methods of following the phosphate transfer.

Quantitative observations were made by Maizels (1933, 1934) who studied the entry of phosphate into human red blood cells and showed that it was most rapid from acid buffers. He also noted that in blood stored for 11 days at 2° phosphate left the cells more slowly than it entered them (Maizels, 1944). Anderson (1942) had also noted this phenomenon in rabbit erythrocytes suspended in plasma at 37°. He followed the phosphate transfer using ^{32}P and found that entry was three to five times as fast as exit under these conditions. On the other hand, the observations of Booth (1938) suggested a more rapid loss of phosphate from erythrocytes than is given by Anderson (1942) or by the results reported here. Booth (1938) showed that pig cells in which the chloride content had been decreased by shaking with phosphate buffer regained the chloride lost by shaking with saline for the same period. He put this down to entry and exit of phosphate but made no phosphate determinations. If the changes in chloride analyses he observed were in fact due to phosphate-chloride exchanges, the rate of phosphate exit indicated is much higher than is reported here for horse erythrocytes. However, his results may be partially accounted for by bicarbonate-chloride exchanges since his solutions probably contained sufficient dissolved carbon dioxide to account for at least half the observed changes (Kolthoff, 1926; Booth, private communication). Such an interpretation does not affect the conclusions of Booth (1938) on the importance of intracellular chloride in studying carbonic anhydrase activity in red corpuscles.

Phosphate transfer and cell metabolism

There is no evidence in this work that the mechanism of phosphate transfer necessarily involves an enzyme system coupled with the cell metabolism (cf. Halpern, 1936; Eisenman, Ott, Smith & Winkles, 1940). While the well washed cells are still capable of active metabolism, they are isolated from sources of energy (e.g. glucose) and yet show no deterioration in their phosphate-transferring capacity during the protracted experiments described.

SUMMARY

1. A method is described for studying the water shift accompanying the bicarbonate-chloride exchange in horse erythrocytes.

2. Methaemoglobin cells treated with phosphate buffer, pH 6, exhibit normal permeability to bicarbonate and chloride ions as far as can be observed by this method.

3. The apparent destruction of the anion permeability of methaemoglobin cells by acid phosphate buffer (Keilin & Mann, 1941; Keilin & Hartree, 1946) is shown to be due instead to a phosphate-chloride exchange across the cell boundary.

4. The apparent irreversibility of the effect of acid phosphate buffer on methaemoglobin cells (Keilin & Hartree, 1946) is probably due to the slowness with which phosphate leaves the cells compared to the rate at which it enters them.

I wish to thank Prof. D. Keilin, F.R.S., for suggesting this problem to me, and for his guidance and encouragement. I am also indebted to Prof. F. J. W. Roughton, F.R.S., for helpful discussion and criticism. This work was carried out during tenure of a research maintenance grant from the University of Cambridge.

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The Reaction between Metmyoglobin and Hydrogen Peroxide

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Transient complexes with well defined spectroscopic characteristics play an important role in the reaction of the haemoprotein enzymes, catalase and peroxidase, with hydrogen peroxide. Two and sometimes three different complexes can be observed depending on the experimental conditions. For example, Keilin & Mann (1937) obtained two red complexes with peroxidase and hydrogen peroxide, and later Theorell (1941) showed that a green complex preceded the formation of the first red complex.

This system has been studied intensively by Chance (1949*a*, *b*) who extended the observation to systems of catalase and hydrogen peroxide or alkyl hydroperoxides (1949*c*). There is, however, very little information from which structural differences accounting for the various complexes can be inferred. The transient nature of the complexes leads to experimental difficulties.

These difficulties were absent in the case of the complex formed with metmyoglobin (MetMb) and hydrogen peroxide. The existence of a well defined red complex in this system and with haemoglobin too is well established. Keilin & Hartree (1935) found that the formation requires 1 molecule of hydrogen peroxide per haematin iron atom. With reducing agents the complex is reduced back to metmyoglobin or methaemoglobin. As far as is known, only this single complex is formed and because it shares certain properties with the secondary complexes of peroxidase and catalase, a detailed knowledge of its behaviour is valuable for the further elucidation of enzyme reaction mechanisms.

In this paper experiments are described on the formation of the metmyoglobin-hydrogen peroxide complex over a wide pH range which reveal that only between pH 8 and 9 is the formation unattended by oxidative side reactions affecting the porphyrin ring. Reduction of the complex with ferrocyanide shows it to have 1 oxidizing equivalent, and magnetic measurements suggest that it may be a compound containing quadrivalent iron. A preliminary account of these experiments has been published (George & Irvine, 1951).

MATERIALS AND METHODS

Metmyoglobin. The metmyoglobin (MetMb) used in these experiments was obtained from horses' hearts, the method of preparation following that of Theorell (1932). Briefly, the minced hearts are extracted with water at 0° and muscle globulins removed by addition of basic lead acetate to the extract, the pH of which has been previously adjusted to 7·0 with 1 N-NaOH. The excess Pb is removed as phosphate with Na_3PO_4, and haemoglobin precipitated by adding $(NH_4)_2SO_4$ to 75% saturation. MetMb is precipitated when the solution is saturated to 100% with $(NH_4)_2SO_4$ and is taken up in the minimum quantity of 3 M-phosphate buffer, pH 6·8. Phosphate is removed by dialysis against distilled water and MetMb again salted out. It is then taken up in 0·6 M-phosphate buffer, pH 5·7, passed through a Seitz filter and stored at 0°.

Quantities of this stock solution were thoroughly dialysed against distilled water before use and the concentration determined following the method of de Duve (1948).

Hydrogen peroxide. Pure 97% (w/w) H_2O_2, free from inhibitors, was used. This was kindly supplied by Laporte Chemicals Ltd. Before each experiment a stock solution (approx. 0·1 N) was standardized against $KMnO_4$ and then diluted to the desired concentration.

Buffer solutions. In the pH range 5·8–7·0 phosphate buffers (Green, 1933) were used. For the pH range 7·8–10·0 borate buffers were used. Experiments at pH 11·1 were carried out in a glycine buffer, while at higher pH values dilute alkali was used. The pH values of all buffer solutions were measured on a Cambridge pH meter.

Methods

Colorimetric or spectrophotometric determinations of optical density were made in the majority of the experiments. Because of its easier and more rapid application, the

former method was used wherever possible (pH range 5·8–9·0). Yellow filters were found to give the most suitable wavelength region. In the more alkaline regions and also in experiments requiring a complete analysis of spectra in the visible and Soret regions, spectrophotometric measurements were made using a Unicam quartz spectrophotometer.

Owing to the relative slowness of the formation and reduction of the H_2O_2-MetMb complex at the concentrations used and especially in the alkaline region, it was necessary to follow the reactions kinetically in order to ascertain when full formation or reduction had taken place.

The percentage complex formation or reduction was calculated by interpolation using Lambert and Beer's law. The optical density of a solution of MetMb and H_2O_2 was found to reach a maximum value when a slight excess of H_2O_2 was present, e.g. a molar ratio between 1·5 and 2·5 to 1 depending on the pH of the solutions. This optical density was taken to be that corresponding to 100% complex formation. The procedure is justified by the experiments of Keilin & Hartree (1950), who found that H_2O_2 concentrations in great excess (100 to 1) were required before appreciable reaction of H_2O_2 and the complex itself occurred.

For the magnetic measurements a modification of the Theorell (1942) microbalance was used. The calibration was carried out with ferrous ammonium sulphate solutions.

RESULTS

Qualitative experiments indicated that a reddish violet complex was formed throughout the pH range 5·0–12·0 when H_2O_2 was added to MetMb. The

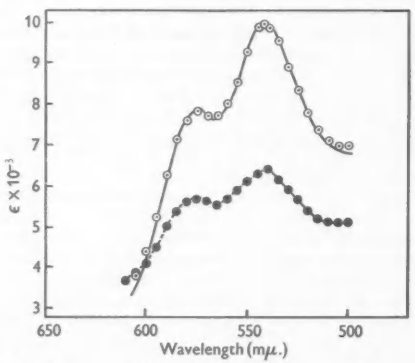

Fig. 1. Recovery of MetMb from complex. Azide spectra; smooth curve represents MetMb + azide; ⊙, reduced complex at pH 8·4 + azide; ●, reduced complex at pH 11·1 + azide.

complex is comparatively stable. Between pH 8 and 9 slight decomposition could be detected at the end of half an hour after formation and complete destruction was apparent after about 48 hr. standing at room temperature. The end product was mainly MetMb. The rate of destruction was more rapid in acid solution.

The complex is a good oxidizing agent and will oxidize ascorbic acid, quinol, *p*-phenylenediamine, leuco-malachite green, iodide ion (but not bromide) and ferrocyanide ion, itself being reduced to MetMb. The rate of oxidation is dependent on pH and is faster in more acid solutions.

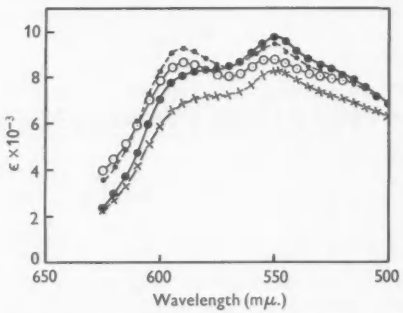

Fig. 2. Effect of pH on complex. Visible region 650–500 mμ. ●, complex at pH 8·4; ×, complex at pH 10·0; •, complex at pH 6·8; ⊙, complex at pH 5·8.

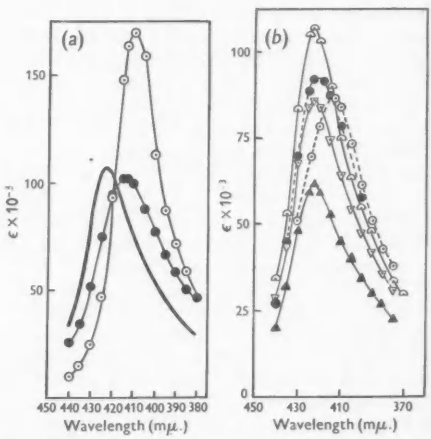

Fig. 3. Effect of pH on complex. Soret region; (*a*) smooth curve represents complex at pH 8·4; ⊙, MetMb, pH 6·8; ●, MetMb, pH 11·1. (*b*) ⌓, complex at pH 8·4; ▽, complex at pH 10·0; ▲, complex at pH 11·1; ●, complex at pH 6·8; ⊙, complex at pH 5·8.

Experiments show that between pH 8·0 and 9·0 the reaction between H_2O_2 and MetMb is straightforward in the sense that the minimum amount of side reactions occur. Thus it is possible within this region to recover 90–100% of MetMb from the complex by the addition of mild reducing agents. By comparison only about 65% MetMb is recoverable at pH 11·1 (Fig. 1). Below pH 8·0 and especi-

ally at pH values $<7{\cdot}0$ side reactions occur producing one or more compounds with strong absorption in the red. Above pH 9·0 the products of side reactions have no such absorption. Thus in Figs. 2 and 3, which show the spectra of the complex in the visible and Soret region, it will be seen that the difference between the spectrum of the complex at pH 10·0 and at pH 8·4 is mainly a difference in intensity. Table 1 gives the millimolar extinction coefficients of the complex at 549 and 423 mμ. at pH values 8·3, 10·0 and 11·1.

On the other hand, there is a definite shift of the band in the Soret region when the complex is formed at a pH $<8{\cdot}0$; at pH 6·8 the band is at 420–423 mμ. and at pH 5·8 it is at 413 mμ. (Fig. 3*b*). In the visible region the difference is borne out by the appearance of a new band at 590 mμ. (Fig. 2).

Table 1. *Millimolar extinction coefficient* ($\epsilon \times 10^{-3}$) *of complex at* 549 *and* 423 mμ. *at different* pH *values*

pH	$\epsilon \times 10^{-3}$ at 549 mμ.	$\epsilon \times 10^{-3}$ at 423 mμ.
8·4	9·8	107·0
10·0	8·3	87·0
11·1	6·5	62·0

Table 2. *Effect on extinction and position of peak of changing the* pH *after complex formation at a given* pH

(*a*) Complex formed at pH 6·8

pH	Position of peak (mμ.)	$\epsilon \times 10^{-3}$
6·8	420–423	93·0
9·0	420–423	94·0
11·1	420–423	94·0

(*b*) Complex formed at pH 9·0

pH	Position of peak (mμ.)	$\epsilon \times 10^{-3}$
9·0	423	102·0
6·8	423	100·0
11·1	423	101·0

An interesting observation on this effect of pH is that once the complex is formed changing the pH seems to have little influence on the spectrum. Table 2*a*, *b* shows some of the results obtained from measurements in the Soret region. This shows that the influence of different hydrogen-ion concentration is exerted during the formation reactions and the effect is not due to a change in spectrum of the complex as the pH is varied.

The nature of the side reactions is not fully understood. It would seem that the reduction of the intensity, which is observed when the complex is formed at a pH $>9{\cdot}0$, is due to varying amounts of cholemyoglobin formed. Thus the carbon monoxide-haemochromogen (obtained by reducing the complex at pH 11·1 with $Na_2S_2O_4$ in the presence of carbon monoxide and adding 20% alkali, cf. Lemberg, Legge & Lockwood (1941)) shows, in addition to the bands at 542 and 573 mμ. of the true haemochromogen, a band at 630 mμ. There is also a considerable reduction of the bands at 542 and 573 mμ. (see Fig. 4). At pH 6·8 and 5·8,

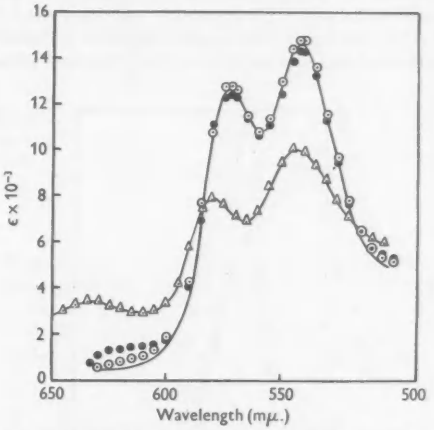

Fig. 4. Haemochromogen spectra, 650–500 mμ., from complex formed at pH $>8{\cdot}0$; smooth curve is haemochromogen from MetMb; ⊙, from complex formed at pH 8·6; ●, from complex formed at pH 8·0; △, from complex formed at pH 11·1.

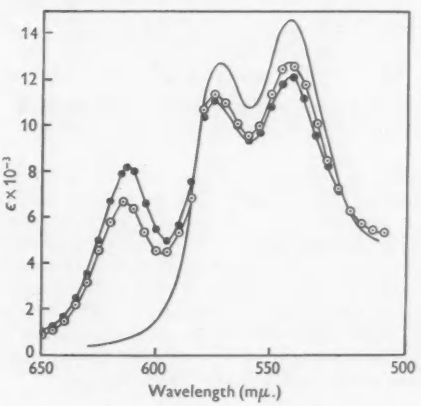

Fig. 5. Haemochromogen spectra, 650–500 mμ. from complex formed at pH $<8{\cdot}0$. Smooth curve is haemochromogen from MetMb; ⊙, from complex formed at pH 6·8; ●, from complex formed at pH 5·8.

however, a different band appears at 615 mμ., and there is very little reduction in the bands of the true haemochromogen (Fig. 5). In addition, the Soret spectrum of the haemochromogen obtained from the complex formed at pH 5·8 is even more intense than that of the true haemochromogen

(Fig. 6). Substitution of the porphyrin ring without ring fission seems a likely explanation.

The following observations show that these side reactions occur during the actual formation of the complex. If MetMb is allowed to stand with the preformed complex for about 1 hr. at either pH 5·8 or 11·1, the haemochromogen spectrum obtained from the mixture is the same as that produced when

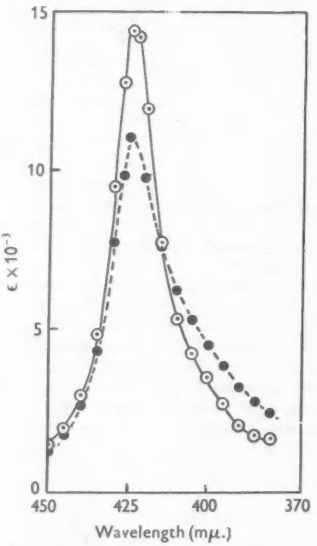

Fig .6. Haemochromogen spectra. Soret region; ●, from MetMb; ⊙, from complex formed at pH 5·8.

MetMb is present with the complex for a few minutes. This suggests that an oxidizing entity more powerful than the complex itself is present during the formation of the complex. Qualitative experiments with luminol support this. If luminol is added to the complex a dull luminescence is obtained, whereas if luminol and MetMb are mixed and H_2O_2 then added (under conditions where the complex is formed in a few seconds) a bright flash is observed followed by a dull luminescence.

Quantitative measurements on the formation of the complex

The formation of the complex between pH 8·0 and 9·0 is characterized by the fact that a 1:1 ratio of H_2O_2 to MetMb does not produce 100% complex formation even in a concentration of MetMb as high as 10^{-4} M. At this concentration 80–90% of complex is produced, the percentage varying between these limits for different samples of MetMb.

The effect of concentration on the formation is only very slight over a tenfold change of absolute concentration and rules out the possibility of an equilibrium of the type

$$\text{MetMb} + H_2O_2 \rightleftharpoons \text{complex}.$$

The results of the experiments are shown in Fig. 7.

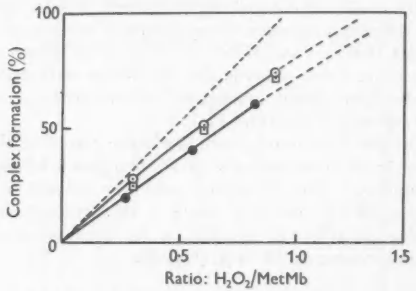

Fig. 7. Effect of concentration on complex formation. ⊙, MetMb $1{\cdot}0 \times 10^{-4}$ M; ⊡, MetMb $4{\cdot}9 \times 10^{-5}$ M; ●, MetMb $8{\cdot}8 \times 10^{-6}$ M. Broken line is theoretical curve based on a 1:1 ratio of H_2O_2 to MetMb.

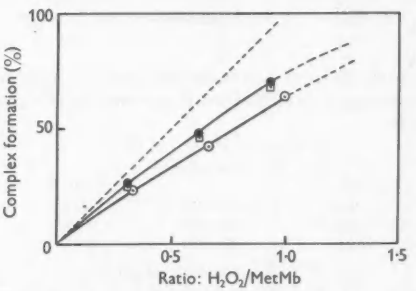

Fig. 8. Effect of pH on complex formation. Broken line is theoretical curve based on a 1:1 ratio of H_2O_2 to MetMb. ●, pH 8·0, MetMb $4{\cdot}7 \times 10^{-5}$ M; ⊡, pH 8·4, MetMb $5{\cdot}0 \times 10^{-5}$ M; ⊙, pH 9·0, MetMb $4{\cdot}8 \times 10^{-5}$ M.

Fig. 8 shows the effect of pH on complex formation which is small within the pH region 8·0–9·0. Although in the figure the maximum complex formation occurs at about pH 8·0–8·4, the pH at which maximum formation is observed appears to differ to a small extent for different samples of MetMb. In none of the samples used, however, was this pH outside the range 8·0–9·0.

Reduction of the complex

Experiments with ferrocyanide ion as the reducing agent show that 1 mole of ferrocyanide will reduce 1 mole of complex completely to give MetMb, within the pH region 8·0–9·0. Typical results of these experiments are shown in Table 3*a*, *b*.

If, instead of adding ferrocyanide to the preformed complex, the ferrocyanide is first added to MetMb in varying amounts and hydrogen peroxide

Table 3. *Reduction of the metmyoglobin-hydrogen peroxide complex by ferrocyanide*

(*a*) pH 8·0

Initial complex (μM)	Ferrocyanide (μM)	Observed complex left (μM)	Complex reduced (μM)	Ratio: $\frac{Fe(CN)_6^{4-}}{Complex}$
24	10	14	10	1·00
24	15	10	14	1·07
24	24	1	23	1·04
40	30	9	31	0·97

(*b*) pH 8·4

Initial complex (μM)	Ferrocyanide (μM)	Observed complex left (μM)	Complex reduced (μM)	Ratio: $\frac{Fe(CN)_6^{4-}}{Complex}$
12	10	2	10	1·00
30	30	2	28	1·07

Table 4. *Reaction of metmyoglobin with hydrogen peroxide in the presence of ferrocyanide at* pH 8

(In these experiments ferrocyanide was added before hydrogen peroxide.)

(*a*) Initial MetMb and H_2O_2 concentrations $1·04 \times 10^{-4}$M and $1·01 \times 10^{-4}$M respectively

$Fe(CN)_6^{4-}$ (μM)	Complex formed (μM)	Oxidizing equivs. of H_2O_2 accounted for (μM)*	(%)
0	91	91	45·0
120	72	192	95·0
140	56	196	97·0
160	38	198	98·0

* On the basis 1 mole complex = 1 mole $Fe(CN)_6^{4-}$ = 1 equiv. H_2O_2. 202 equivs. present initially.

(*b*) Initial MetMb and H_2O_2 concentrations $5·2 \times 10^{-5}$M and $5·0 \times 10^{-5}$M respectively

$Fe(CN)_6^{4-}$ (μM)	Complex formed (μM)	Oxidizing equivs. accounted for (μM)*	(%)
0	37	37	37·0
60	29	89	89·0
70	23	93	93·0
80	14	94	94·0
90	5	95	95·0

* On the basis 1 mole complex = 1 mole $Fe(CN)_6^{4-}$ = 1 equiv. H_2O_2. 100 equivs. present initially.

then introduced, complex formation is observed even when the amount of ferrocyanide present is in excess of the amount of complex expected. Table 4*a*, *b* gives the results of two typical experiments of this nature at different absolute concentrations.

Magnetic properties of the complex

Preliminary measurements of the molar magnetic susceptibility of the complex at pH 8·6 indicate that it is about 11 000–11 800 $\times 10^{-6}$ c.g.s. units at room temperature. This corresponds to a magnetic moment of 5·1–5·3 Bohr magnetons (μ_B) and suggests the presence of four unpaired electrons. These figures may be compared with those quoted by Hartree (1946): reduced peroxidase $\chi_m \times 10^{-6}$ c.g.s. units = 11 410; haemoglobin $\chi_m \times 10^{-6}$ c.g.s. units = 12 290, $\mu_B = 5·43$; myoglobin $\chi_m \times 10^{-6}$ c.g.s. units = 12 400, $\mu_B = 5·46$. (See footnote p. 516.)

DISCUSSION

The first significant fact which emerges is the effect of pH on the reaction between hydrogen peroxide and metmyoglobin. The reaction can be said to be 'normal' within the pH range 8·0–9·0. Outside this region there is the peculiarity on the alkaline side of a reduction of the intensity of the complex, and on the acid side the production of a different spectrum (Figs. 2 and 3). The haemochromogen spectrum obtained from the complex formed at pH 11·1 (Fig. 4) shows a peak at 630 mμ. and a reduction of the intensities of the peaks at 542 and 573 mμ. of the true haemochromogen. This is typical of the behaviour which Lemberg *et al.* (1941) associate with choleglobin formation. Moreover, the formation of choleglobin, more specifically in this case of cholemyoglobin, would account for the absence of any marked absorption in the visible and Soret regions other than that attributed to the complex.

On the acid side, however, this explanation will not hold. The haemochromogen spectra obtained from the complex at pH 6·8 and 5·8 show a peak at 615 mμ., not at 630 mμ., and there is little reduction of the peaks at 542 and 573 mμ. (Fig. 5). This fact, together with the observation that in the Soret region there is not only no decrease, but an actual rise in the intensity of the haemochromogen spectrum obtained from the complex at pH 5·8 above the true haemochromogen (as shown in Fig. 6), suggests that a different type of oxidative attack on the porphyrin ring has occurred. This type of attack presumably does not affect the conjugated double-bond system to the same extent that cholemyoglobin formation does, for the intensity of light absorption in the Soret region is believed to be a characteristic of the conjugated system.

The absence of any effect of pH on the spectrum of the complex once it has been formed and the fact that the complex does not apparently react either with itself or added metmyoglobin to give the side reactions, indicate clearly that these side reactions occur during the formation of the complex. The experiments with luminol suggest that a transient oxidizing agent, more powerful than the complex, is produced during the formation reaction.

The data of the other experiments reveal three important features:

(1) The complex is not formed in any set of equilibrium reactions involving metmyoglobin and hydrogen peroxide, either in their ionized or unionized forms, e.g.

$$\text{MetMb} + H_2O_2 \rightleftharpoons \text{complex}.$$

In this respect its formation is unlike that proposed by Chance (1949*b*, 1951) for the peroxidase and catalase complexes, i.e.

$$\ldots FeOH + H_2O_2 \rightleftharpoons \ldots FeOOH + H_2O.$$

This suggests that neither H_2O_2 nor HO_2^- is present in the metmyoglobin complex.

(2) Full formation of the complex is not obtained for a 1:1 ratio of hydrogen peroxide to metmyoglobin. In view of the fact that the system is not an equilibrium system this result must mean that hydrogen peroxide or metmyoglobin or complex (or more than one of these) is disappearing during the reaction. The disappearance of metmyoglobin can be ruled out since increasing the peroxide ratio increases complex formation. Furthermore, within the pH region 8·0–9·0 nearly all the metmyoglobin can be recovered, even though in this region 100% formation is not observed for 1:1 ratio. If complex is being destroyed, the destruction must take place while formation is occurring, since once the complex has been formed no destruction is detectable for about half an hour (within the pH region 8·0–9·0). In the absence of an equilibrium the destruction of complex could only be interpreted as producing some new entity which would be both colourless and reducible to metmyoglobin, to account, first, for the lack of absorption and, secondly, for the complete recovery of metmyoglobin at certain pH values. This does not seem likely.

(3) The oxidizing equivalent of the complex is unity (Table 3). Hence the full oxidizing equivalent of the peroxide can only be accounted for by assuming that some other oxidizing entity also with 1 oxidizing equivalent is produced during the reaction of hydrogen peroxide and metmyoglobin. Evidence supporting this is obtained by adding a reducing agent to the metmyoglobin before introducing hydrogen peroxide when the full oxidizing capacity of the peroxide is realized (see Table 4).

On the basis of this last feature, the equation for the formation of the complex can be written in its simplest form

$$\text{MetMb} + H_2O_2 \rightarrow \text{complex} + X,$$

2 oxidizing equivs.

$\rightarrow$ 1 oxidizing equiv. + 1 oxidizing equiv.

A tentative mechanism accounting for the complex and X each with 1 oxidizing equivalent is the formation of an $OH^{\cdot}$ radical and ferryl myoglobin in a reaction of the type

$$Fe^+(H_2O) + H_2O_2 \rightarrow \underset{\text{(complex)}}{FeO} + \underset{\text{(X)}}{OH} + H_2O + H^+,$$

where Fe^+ represents the haematin iron atom of metmyoglobin. Ferrylmyoglobin by analogy with the hypothetical ferryl ion FeO^{2+} and the well known vanadyl ion VO^{2+} would be a compound of quadrivalent iron and would thus have 1 oxidizing equivalent when reduced to a compound in the ferric state. The identification of the complex with ferrylmyoglobin would be in accordance with magnetic measurements which show the presence of four unpaired electrons.* The electronic structures of the Fe^{2+}, Fe^{3+} and Fe^{4+} ions are given in Fig. 9.

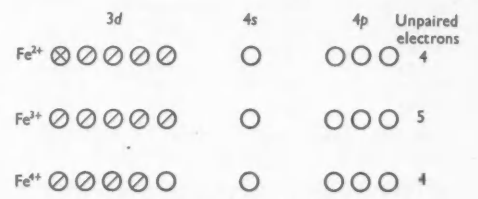

Fig. 9. Electronic structures of Fe^{2+}, Fe^{3+}, Fe^{4+}.

It can be seen that both Fe^{2+} and Fe^{4+} ionic compounds would have four unpaired electrons. But in the case of the MetMb-H_2O_2 complex, this clearly cannot be a ferrous compound for it is a strong oxidizing agent which on reduction yields a ferric compound.

The identification of X with an OH radical fits in with the observations that (1) X is an oxidizing agent. (2) X is transient; only during the actual formation of the complex is its oxidizing ability observed. (3) During the formation of the complex a flash is observed if luminol is present. No flash is

* *Note added in proof:* Theorell & Ehrenburg (1952) have reported a susceptibility value of 3800×10^{-6} c.g.s. units: our more recent experiments show that the high value of about 11000×10^{-6} c.g.s. units quoted above is in error on account of decomposition of the complex during the measurements. We find the correct value to be $< 8400 \times 10^{-6}$ c.g.s. units. A covalent instead of an ionic structure containing quadrivalent iron would be in accord with these new values, for d^2sp^3 covalent bond formation of Fe^{4+} results in two unpaired electrons ($\chi_m = 3390 \times 10^{-6}$ c.g.s. units) as can be seen from Fig. 9.

obtained if luminol is added after the complex has been formed.

The side reactions could be accounted for because of the high oxidizing activity of the OH radical.

SUMMARY

1. Metmyoglobin forms a complex with hydrogen peroxide throughout the pH range 5·0–12·0. Only within the pH range 8·0–9·0 is this reaction unattended by oxidative attack on the porphyrin ring.

2. On the alkaline side the attack on the ring seems to give cholemyoglobin, for a regular decrease in the intensity of the absorption bands is observed both in the visible and Soret regions of the spectrum. In solutions more acid than pH 8·0 a different type of oxidative attack occurs, for no regular decrease in intensity is observed, but there is a shift of the band maxima. These side reactions are not due to the reaction of the complex itself with metmyoglobin, but arise by some other mechanism during the formation of the complex.

3. The system $MetMb + H_2O_2$ is not an equilibrium system. The effect of concentration on the formation of the complex is only very small over a tenfold change of absolute concentration.

4. The oxidizing equivalent of the complex is unity. By adding a reducing agent to the metmyoglobin before peroxide it is possible to account for the two oxidizing equivalents of the peroxide. This indicates the reaction to be of the type

$$MetMb + H_2O_2 \rightarrow complex + X.$$

5. A tentative mechanism is proposed identifying the complex with ferrylmyoglobin, a compound of quadrivalent iron, and X with the OH radical. The high oxidizing activity of the OH radical could account for the oxidative side reactions.

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The Ionization of Acidic Metmyoglobin

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The problem of establishing thermodynamic constants for the ferric complexes of haemoproteins is important in at least two respects. In the first place such constants offer a workable criterion where a parallelism is sought between the behaviour of simple ferric iron and that of the iron atom in the porphyrin ring of haemoproteins. Secondly, a knowledge of such data would offer additional evidence for elucidating hitherto uncertain structures, for instance those of haemoprotein-peroxide complexes, by comparing approximate thermodynamic constants for the formation of these complexes with corresponding data for the formation of, say, the hydroxide, fluoride or cyanide complexes where the structure is better known.

Some equilibrium data on the ionization of acidic methaemoglobin in water, which is equivalent to the formation of the hydroxide complex, have been published by several workers but no values were obtained for ΔH or ΔS. Furthermore, there appears to be an interesting anomaly in the effect of neutral salts on this ionization constant. An inspection of the results of Austin & Drabkin (1935) which were based on spectrophotometric studies of canine methaemoglobin, and those of Coryell, Stitt & Pauling (1937), derived from magnetic measurements on bovine methaemoglobin, shows that this ionization constant falls with increasing ionic strength, i.e. pK moves away from the isoelectric point of the protein, whereas the effect of neutral

salts on titration curves of several proteins, both simple and conjugated, is known to cause the reverse trend (Sørensen, Linderstrøm-Lang & Lund, 1926; Cohn, Green & Blanchard, 1937; Cannan, Palmer & Kibrick, 1942).

This paper deals with the corresponding ionization on the haem iron of metmyoglobin. Metmyoglobin contains only one ferric protoporphyrin (haematin) group per molecule, and it therefore presents a system which is similar to but simpler than that of methaemoglobin. The variation of its ionization constant with ionic strength and temperature is investigated in this paper and values are obtained for pK, ΔH and ΔS. Corresponding work on methaemoglobin is in progress.

Theoretical formulation

Myoglobin is stable in the pH range 6·0–13·0 (Theorell, 1934). Around pH 9·0 the reversible equilibrium between acidic (brown) and alkaline (red) forms of metmyoglobin may be represented by a single ionization of the type

$$HP \rightleftharpoons P + H^+,$$

where HP stands for a molecule of acidic metmyoglobin and P for its conjugate base, the alkaline form. The net electrical charges on these molecules are unspecified, but P will clearly have a charge which is one integral unit less than that of HP. The ionization involves a water molecule occupying the sixth co-ordination position of the octahedral ferric complex (Keilin & Hartree, 1949; Haurowitz, 1949). It may therefore also be represented by

$$Fe^+(H_2O) \rightleftharpoons FeOH + H^+,$$

or by the equivalent relation

$$Fe^+(H_2O) + OH^- \rightleftharpoons FeOH + H_2O.$$

For such an equilibrium

$$K_{HP} = \frac{a_P\, a_{H^+}}{a_{HP}} = a_{H^+} \frac{c_P}{c_{HP}} \frac{f_P}{f_{HP}}, \qquad (1)$$

where for a given solvent and at a given temperature K_{HP} is a true thermodynamic constant. The symbols a, c, f stand for activity, molar concentration and activity coefficient respectively.

Experimentally it is convenient to measure an equilibrium constant K' which is defined by the equation

$$pK' = pH + \log \frac{c_{HP}}{c_P}, \qquad (2)$$

where pH values are measured against an arbitrary standard in a pH meter and the ratio c_{HP}/c_P is measured spectrophotometrically. This empirical constant is related to the true thermodynamic constant by the following equation:

$$pK' = pK_{HP} + (pH + \log a_{H^+}) + \log \frac{f_P}{f_{HP}}. \qquad (3)$$

pK′ is therefore subject to primary salt effects and in the limit of infinite dilution, where all activity coefficients approach unity, the value of pK′ becomes that of the extrapolated quantity pK'_0 such that

$$pK'_0 = pK_{HP} + (pH + \log c_{H^+}).$$

K'_0 may be described as an approximation to the thermodynamic ionization constant, but it cannot be precisely identified with K_{HP} because pH cannot be identified with $-\log a_{H^+}$ or $-\log c_{H^+}$ (Guggenheim, 1930; Bates, 1948). Nevertheless, it has been assumed throughout this work that the measured changes in pH give reasonable values for the corresponding changes in $\log a_{H^+}$. An estimate of the uncertainties involved is made below in the Discussion.

MATERIALS AND METHODS

Preparation of metmyoglobin. Metmyoglobin (MetMb) was prepared from horses' hearts following a modification of the method of Theorell (1932). Details of the procedure are the same as used by George & Irvine (1952). The concentration of a given solution of MetMb was estimated, when required, following the procedure of de Duve (1948), which involves the conversion of MetMb into the carboxymyoglobin complex in a 0·067 M-Na_2HPO_4 buffer. de Duve's absorption spectrum for this complex was used as a standard.

Buffer solutions. Borate buffers were used throughout except in the few cases shown in Table 1 and Fig. 1 where veronal buffers were used to investigate specific salt effects. To cover the pH range 7·8–10·2 borate buffers were made from mixtures of boric acid and NaOH, and veronal buffers over the pH range 7·8–9·2 from sodium diethylbarbiturate and HCl. All solutions were prepared from A.R. materials. Ionic strengths were calculated from the contribution of Na^+ and $H_2BO_3^-$ ions (or Na^+ and barbiturate ions) and the buffer solutions were adjusted to the same total ionic strength with NaCl. Normally, these buffer mixtures were made from 0·1 M solutions and had ionic strength (I) adjusted to 0·041. However, for work at very low salt concentrations, stock buffers were made with CO_2-free water and adjusted to $I = 0{\cdot}018$; for lower ionic strengths these were further diluted with the same water. The contribution to I by the haemoprotein and traces of associated salt is discussed later in this paper.

Reference solutions of acidic and alkaline metmyoglobin were made, respectively, in a KH_2PO_4-NaOH buffer of pH 6·0 and a glycine-NaOH buffer of pH 11·1 and, as was known from preliminary measurements, these were taken to represent 99·5% conversion into acidic or alkaline form respectively.

Spectrophotometric measurements. In a given buffered solution of MetMb, usually about 5×10^{-5} M, the fraction present as alkaline (alk.) MetMb was determined by comparing the optical absorption of the solution with the corresponding absorptions of the reference solutions of acid MetMb and alk. MetMb containing the same concentration of MetMb. A Unicam quartz spectrophotometer was used for measuring optical densities. This method of analysis assumes the validity of both Lambert's and Beer's Laws and also neglects the specific effects of ions in the solution on the absorption of light. The applicability of this method at ionic

strengths below 0·01 has been verified by previous workers, for instance Halban & Körtum (1934). It is also known that the molecular extinction coefficient of an absorbing ion is not constant at high salt concentrations (Halban & Eisenbrand, 1928), but as the measurements reported in this paper refer to differences and not absolute quantities this effect could be neglected, especially so because the region investigated had $I<0{\cdot}7$. The question of specific salt effects on the equilibrium is dealt with elsewhere in this paper.

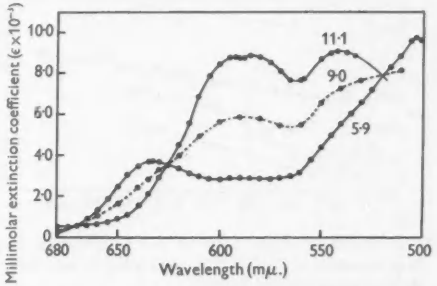

Fig. 1. Absorption spectra of metmyoglobin (MetMb) in the visible range 680–500 mμ. at 20°. Concentration, $5{\cdot}0\times10^{-5}$M. $I=0{\cdot}01$. Spectrum at pH 5·9 represents nearly 100% acid MetMb, that at pH 11·1 nearly 100% alk. MetMb and the intermediate one at pH 9·0 nearly 50% of each.

The absorption spectra for the brown acid MetMb and red alk. MetMb in the visible range 680–500 mμ. are quite similar to the corresponding ones of MetMb. In Fig. 1 three such spectra are shown for a $5{\cdot}0\times10^{-5}$M solution of MetMb at $I=0{\cdot}01$; the one at pH 5·9 represents the acidic form, that at pH 11·1 represents the alkaline form, and the intermediate one is at pH 9·0. The general shape of this intermediate spectrum and particularly the fact that it passes through three isobestic points strongly support the original assumption that the system consists of two species only (acidic and alkaline forms). At the wavelength 582 mμ. acid MetMb has a millimolar extinction coefficient $\epsilon\times10^{-3}=2{\cdot}9_2$ while that of alk. MetMb is $8{\cdot}7_1$. This wavelength was chosen for comparing optical densities and all measurements were made using 1 cm. glass cells, the solutions being read against distilled water as a blank.

During the measurement of optical density it was possible to control temperatures to $\pm0{\cdot}2°$ using the following simple methods. For measurements above room temperature the spectrophotometer cell-holder with the cells and solution in it was warmed slowly on an electric hotplate to the temperature required, and it was found that subsequent cooling during the actual measurement of optical density did not exceed the limit indicated above. For measurements below room temperature water was circulated first through an ice-water mixture and then through two brass boxes fitted tightly in the cell-holder on either side of the cells.

Measurement of pH. pH was measured directly with a Cambridge pH meter using a glass electrode and a 0·05M solution of A.R. borax in CO_2-free water as standard. To test the reliability of the instrument the pH of this standard (9·18 at 20°) was checked against that of a 0·05M-potassium hydrogen phthalate buffer (3·99 at 20°). The discrepancy did not exceed 0·01 unit of pH; the degree of uncertainty involved in the measurement of pH by this method is referred to in the discussion. In the early stages of the investigation the pH of buffer alone was measured. A study of the salt effect on the pH of borate buffers at 20° showed that in the low ionic strength region the results were in reasonable agreement with a Debye-Hückel limiting law, and it was therefore assumed that the method of measurement was good enough. All later measurements were done on the actual buffered solution of MetMb directly after the measurement of optical density. In the case of very dilute buffers, where CO_2 effects become significant, the simultaneous measurement of optical density and pH was carried out with the help of an assistant immediately after the preparation of the solutions. Invariably it took under 2 min. for electrodes and solution to come to equilibrium. In this time the contribution to I made by KCl diffusing from the bridge, as assessed from conductimetric measurements, is inappreciable and was therefore ignored. Further, this KCl bridge was frequently renewed in order to ensure a constant potential at the boundary junction with the solution. During an actual measurement of pH the temperature of the solution was accurately controlled in a thermostat.

Sample run. Temp. $=37{\cdot}0\pm0{\cdot}2°$. Stock solution of MetMb in water $=4{\cdot}0\times10^{-4}$M. Reference solutions: (i) Acid MetMb containing 2·5 ml. stock solution + 18·0 ml. buffer solution (KH_2PO_4 + NaOH, pH 6·0). (ii) Alk. MetMb containing 2·5 ml. stock solution + 18·0 ml. buffer solution (glycine + NaOH, pH 11·1). Solutions or measurements made in H_3BO_3-NaOH buffers of pH values 8·6, 8·8, 9·0, 9·2, 9·4 (all adjusted to $I=0{\cdot}041$) containing 2·5 ml. stock MetMb + 3·0 ml. of 2M-NaCl + 15·0 ml. buffer solution. Calculated $I=0{\cdot}32$. For results see Table 1.

Table 1. *Preliminary experiment to demonstrate the validity of the optical method to determine* pK′

	pH (measured)	Optical density (measured at 582 mμ.)	Alk. MetMb (%)	pK′ using eqn. 2
Reference acid MetMb	—	0·143	Taken as 0·5	—
Reference alk. MetMb	—	0·427	Taken as 99·5	—
Solution in pH 8·6 buffer	8·29	0·210	24·1	8·79
Solution in pH 8·8 buffer	8·49	0·242	35·1	8·76
Solution in pH 9·0 buffer	8·69	0·267	44·0	8·79
Solution in pH 9·2 buffer	8·90	0·302	55·9	8·80
Solution in pH 9·4 buffer	9·11	0·333	67·0	8·80

Mean pK′ $=8{\cdot}79\pm0{\cdot}01$.

RESULTS

The above procedure was used in determining pK′ over a range of ionic strengths and temperature. The results are given in Table 2, every pK′ value being the arithmetic mean of some four determinations. The region of very low ionic strengths was investigated at two temperatures only, 20 and 37°. Fig. 2 shows the variation of pK′ with $\sqrt{I}$ and temperature; the important point it illustrates is the reversal of the salt effect below $\sqrt{I}=0{\cdot}1$. Table 2 and Fig. 2 also show that pK′ values obtained with veronal buffers fall within the limits of experimental error on pK′ obtained with borate buffers. Although this was taken to suggest that no marked specific salt effects interfered with our measurements it does not preclude the possibility of specific interaction between electrolyte ions and the protein (cf. Adair & Adair, 1934; Sidwell, Munch, Barron & Hogness, 1938).

Table 2. *Effect of buffer, neutral salts and temperature on the ionization of acidic metmyoglobin (acid MetMb) in water,* $Fe^{+}(H_2O)=FeOH+H^{+}$, *measured within the* pH *range* 7·8–9·2

(I = ionic strength calculated from contribution of buffer alone. $pK'=pH+\log\frac{(\text{acid MetMb})}{(\text{alk. MetMb})}$ as in eqn. 2.)

Temperature (°)	Buffer	I (calc.)	pK′
7·5±0·2	Borate	0·037	9·12±0·01
		0·117	9·14±0·01
		0·284	9·18±0·00
20·0±0·2	Borate	0·001	8·95±0·01
		0·002	8·93±0·02
		0·003	8·91±0·02
		0·004	8·89±0·01
		0·009	8·89±0·01
		0·027	8·90±0·00
		0·080	8·92±0·00
		0·173	8·95±0·01
		0·224	8·96±0·01
		0·399	8·98±0·01
		0·556	9·01±0·01
		0·698	9·03±0·01
	Veronal	0·044	8·93±0·02
	Veronal	0·286	8·99±0·02
28·0±0·2	Borate	0·037	8·80±0·01
		0·088	8·85±0·01
		0·166	8·87±0·01
		0·315	8·92±0·01
	Veronal	0·044	8·81±0·02
	Veronal	0·286	8·90±0·03
37·0±0·2	Borate	0·001	8·72±0·01
		0·002	8·69±0·02
		0·004	8·68±0·01
		0·005	8·65±0·01
		0·009	8·65±0·02
		0·037	8·67±0·01
		0·088	8·72±0·01
		0·166	8·75±0·01
		0·315	8·79±0·01

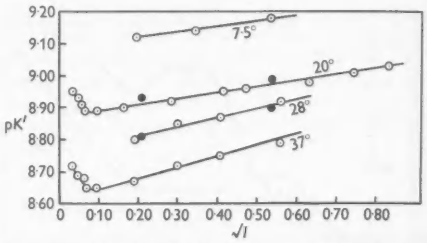

Fig. 2. Variation of pK′ with $\sqrt{I}$ and temperature taken from the data of Table 1. ⊙, runs using borate buffers; ●, runs using veronal buffers.

The values given in Table 3 for the approximate thermodynamic ionization constant pK'_0 were obtained by linear extrapolation in the low ionic strength region after a correction term 0·001 was added to the calculated ionic strengths. This, as will be shown below, is an estimate of the contribution to I from the effective charge on the haemoprotein molecule. Now the limits of uncertainty in the results are such that the plots of pK′ against I, $\sqrt{I}$, $\frac{\sqrt{I}}{1+\sqrt{I}}$ and $\frac{\sqrt{I}}{1+2\sqrt{I}}$ all appear tolerably linear in this low ionic strength region. Four sets of pK'_0 values are therefore given in Table 3, and one of these extrapolations is illustrated in Fig. 3. In all these cases it was found that the lines at 20 and 37° are approximately parallel, their slopes, excluding that of the plot against I, ranging from $-2{\cdot}3$ to $-2{\cdot}9$.

In the higher ionic strength region it was found that pK′ plots were also linear with $\sqrt{I}$ as well as with I. Linear extrapolations of these plots give respectively pK′ ($\sqrt{I}\rightarrow 0$) and pK′ ($I\rightarrow 0$) which have no thermodynamic significance, but it is interesting to note that their temperature coefficients, shown in Fig. 4, are both linear.

Table 3. *Values for the approximate thermodynamic ionization constant* pK'_0 *of acidic metmyoglobin obtained by linear extrapolation of* pK′ *plots against* I, $\sqrt{I}$, $\frac{\sqrt{I}}{1+\sqrt{I}}$ *and* $\frac{\sqrt{I}}{1+2\sqrt{I}}$ *in the region of* $I<0{\cdot}01$

(One of the plots is illustrated in Fig. 3.)

Temperature (°)	$I\rightarrow 0$	$\sqrt{I}\rightarrow 0$	$\frac{\sqrt{I}}{1+\sqrt{I}}\rightarrow 0$	$\frac{\sqrt{I}}{1+2\sqrt{I}}\rightarrow 0$
20·0±0·2	8·995	9·020	9·065	9·070
37·0±0·2	8·765	8·790	8·825	8·830

The results referred to above were all obtained from measurements within the pH range 7·8–9·2 and, as can be seen from Table 2, pK′ was invariably found constant with an experimental scatter not exceeding ± 4 %. However, when solutions of metmyoglobin in buffer outside this pH range were

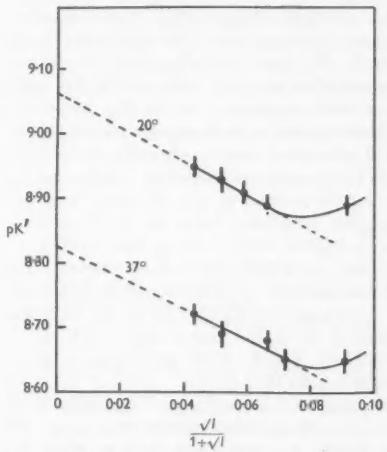

Fig. 3. Illustrating the method of obtaining the extrapolated constant pK'_0. Plots at 20 and 37° of the measured ionization constant, pK′, against the function $\frac{\sqrt{I}}{1+\sqrt{I}}$, using corrected values of I and showing the limits of experimental scatter in the results.

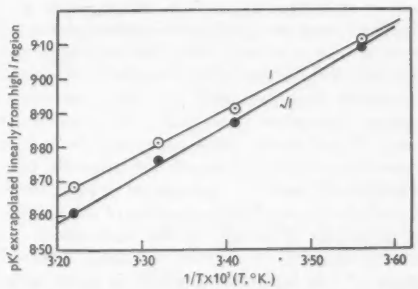

Fig. 4. Temperature coefficients of the 'hypothetical' ionization constants obtained by linear extrapolation of the plots of pK′ against $\sqrt{I}$, ●, and I, ⊙, from the region corresponding to $I > 0{\cdot}01$.

used pK′ was found to be no longer constant and its value rose steadily with increasing pH. The effect was observed both below pH 7·8 and above pH 9·2 and is illustrated in Fig. 5 where $\log \frac{\text{(alk. MetMb)}}{\text{(acid MetMb)}}$ is plotted against pH over the range 8·2–10·2. For a 'normal' run below pH 9·2 this plot is linear and with a slope of unity. The plots above pH 9·2 are very slightly curved and have mean slopes which are evidently values of n in the equation:

$$\log \frac{\text{(alk. MetMb)}}{\text{(acid MetMb)}} = \text{const.} + n\,.\text{pH}. \qquad (4)$$

These values of n are fractional and approach unity with increasing ionic strength of solution. Further comment on this result is made below in the discussion.

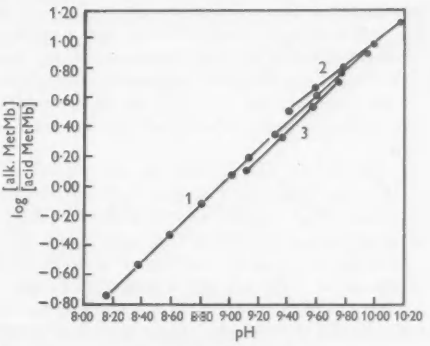

Fig. 5. Plots of $\log \frac{\text{(alk. MetMb)}}{\text{(acid MetMb)}}$ against pH, the linear slopes of which are the values of n in eqn. 4. Experimental details as described in the text, borate buffers being used. Temp. 20°. Run (1): $I = 0{\cdot}11$; section below pH 9·2 gives $n = 1{\cdot}00$ (normal run), section above pH 9·2 gives $n = 0{\cdot}80$. Run (2): $I = 0{\cdot}02$; pH range 9·4–10·2, $n = 0{\cdot}75$. Run (3): $I = 0{\cdot}32$; pH range 9·1–9·8, $n = 0{\cdot}92$.

Calculation of ΔH° and ΔS°

An approximate value for the change in heat content accompanying the ionization can be obtained by integrating the van't Hoff equation

$$\left(\frac{\mathrm{d} \ln K}{\mathrm{d}T}\right)_p = \frac{\Delta H^\circ}{RT^2},$$

if it is assumed that ΔH° is constant over the range of temperature investigated. On this assumption the values for pK'_0 at 20 and 37° given in Table 3 lead to a mean $\Delta H^\circ = 5{\cdot}75 \pm 0{\cdot}67$ kg. cal./mole. It is interesting to compare this result with other estimates of ΔH. Thus the minima of the pK′/$\sqrt{I}$ plots at 20 and 37° give ΔH roughly as 6·1 kg. cal./mole, while the temperature coefficients shown in Fig. 4 lead to ΔH values of 6·5 and 5·7 kg. cal./mole.

The entropy change accompanying this ionization may now be obtained at, say, 20°. Table 3 gives mean pK'_0 at 20° as $9{\cdot}038 \pm 0{\cdot}030$, therefore the free energy change for the process is

$$\Delta G^\circ = -RT \ln K = 12{\cdot}13 \pm 0{\cdot}04 \text{ kg. cal./mole},$$

therefore

$$\Delta S^\circ = \frac{\Delta H^\circ - \Delta G^\circ}{T} = -21{\cdot}7 \pm 2{\cdot}4 \text{ cal./mole/degree}.$$

DISCUSSION

The results outlined above indicate that the ionization of acid metmyoglobin, which probably involves the releasing of a proton from the water molecule attached to the haem iron, is an endothermic process which is markedly influenced by neutral salts. The addition of sodium chloride was found to favour this ionization up to ionic strengths of about 0·01, but further addition of salt repressed the ionization. In view of the uncertainty of the term $(pH + \log a_{H^+})$ in eqn. 3 it is not possible to discuss this effect adequately in terms of the general effect of salts on activity coefficients, though it is easy to interpret the reversal of the salt effect qualitatively in terms of a Debye-Hückel extended equation.

It was stated above that in the region with $I < 0{\cdot}01$ our data fitted linear plots against four functions of I all equally well. Previous workers in the allied fields of solubility and dissociation curves of proteins have used a variety of Debye-Hückel equations as well as the zwitterion model of Scatchard & Kirkwood (1932) in discussing activity coefficients of protein ions. This may be illustrated by referring to the discussions of Cohn & Prentiss (1927), Green (1932), Cannan (1938) and Grönwall (1942). We have given our results in Table 2 in four sets corresponding to four modes of extrapolation without committing ourselves on the question of the theoretical model. Taking $-2{\cdot}6$ as the mean slope of the linear plots, and assuming that a Debye-Hückel limiting law is applicable to such dilute solutions of haemoprotein and electrolyte, the ionization is found to correspond roughly to the charge types $z_{HP} = -2$ and $z_P = -3$ (if these charge types are considered tolerably constant over this linear range). The result implies that as far as this ionization is concerned the molecule behaves as though it had a small effective negative charge. Since the net negative charge on a metmyoglobin molecule around pH 9 is almost certain to be large, the above result suggests that most of the charged groups in the molecule are, as it were, frozen at large distances from the haem iron atom. This argument also illustrates how difficult it is to assess the contribution of metmyoglobin to the total ionic strength of the solution. We have reckoned that our solutions, usually about 5×10^{-5}M-metmyoglobin, do not contribute more than 0·001 to I, i.e. 20 equiv. per mole (cf. Cannan, 1938).

Further to this ambiguity in assigning values to I, the following sources of error in our results may be mentioned. The effect of variation in liquid junction potential on the calibration of the pH scale cannot be estimated with confidence, but our pH measurements are probably precise to ± 3 or 4% (Edsall, 1943; Bates, 1948) and the spectrophotometric measurements to ± 1%. The error in pK′ is therefore ± 4 or 5% which is seen to cover all experimental scatter around the mean pK′ values of Table 2, and due to the uncertainty about the mode of extrapolation ΔH° should be uncertain to about ± 12% and this leads to a $\Delta S^\circ = -21{\cdot}7 \pm 2{\cdot}4$ cal./mole/degree.

This entropy change could arise not only from changes in charge and structure involving the haem Fe, H_2O and OH^- but also from structural changes within the protein molecule. For instance, Eley (1943) suggested that in the dissociation of oxyhaemoglobin such changes occurred within the 17 000 molecular weight sub-units of the molecule. Actually the value obtained above for ΔS accompanying the ionization of acidic metmyoglobin can be roughly accounted for in terms of contributions from Fe, H_2O and OH^- without taking into consideration any contribution from the protein. Despite the great measure of uncertainty of such a calculation we consider it justifiable to conclude that the ionization of acidic metmyoglobin is a normal equilibrium which does not involve structural changes within the protein.

As mentioned previously, the results quoted in Tables 2 and 3 were all obtained from measurements done within the pH range 7·8–9·2. Since the net charge on a metmyoglobin molecule depends on the pH it was rather surprising to find pK′ constant. In terms of eqn. 2, this means that no appreciable interaction between this equilibrium and those of, say, other acidic groups in the molecule occurs within this pH range. Fig. 5 illustrates the behaviour at higher pH in the region which corresponds to over 80% alkaline metmyoglobin. The value of n in eqn. 4 is fractional and approaches unity as the ionic strength increases. Although the theoretical interpretation of the phenomenon remains somewhat uncertain (cf. Linderstrøm-Lang, 1924; Cannan, 1938) this could be a case of interacting equilibria caused, for instance, by the ionization of ϵ-amino groups of lysine residues which form a considerable fraction of the protein in the molecule (Tristram, 1949) and which start ionizing about pH 9·4 (Cohn & Edsall, 1943). The extent of this interaction, which is given quantitatively by the value of n, is small when compared with that which occurs in the haemoglobin-oxygen reaction (cf. Roughton, 1949) and there is the further difference that in the metmyoglobin ionization interaction leads to a decrease in the equilibrium constant, that is the groups interact in such a way as to weaken one another (Wyman, 1948), whereas with haemoglobin the effect of interacting haems is to increase the apparent overall equilibrium constant. This is the physical significance of the fractional values of n in the metmyoglobin case. The effect of increasing ionic strength may be thought of

either in the terms of a general salt effect, or in terms of specific interaction between the ions in solutions and the charged groups in the protein, with subsequent suppression of this effect.

SUMMARY

1. The equilibrium constant for the ionization of (horse) acid metmyoglobin which may be represented as $Fe^+(H_2O) = FeOH + H^+$ has been measured over a range of pH, ionic strength and temperature.

2. The effect of salts on this equilibrium is marked. Thus addition of sodium chloride favours the ionization up to an ionic strength of about 0·01 but beyond this the effect is reversed. Extrapolation to zero ionic strength gives $pK'_0 = 9·038 \pm 0·030$ at 20° and $8·803 \pm 0·025$ at 37°. From the equilibrium constant at two temperatures the ionization is found to be endothermic to the extent of $5·75 \pm 0·67$ kg. cal./mole. $\Delta S^\circ_{20} = -21·7 \pm 2·4$ cal./mole deg.

3. It is only possible to measure this ionization constant within the pH range 7·8–9·2. In more alkaline or acidic solutions significant interaction takes place between this equilibrium and, presumably, other ionizations in the molecule, and the value of the ionization constant falls steadily with increasing pH. This effect is suppressed at high ionic strengths which suggests specific interaction between electrolyte ions in solution and the charged groups on the protein.

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The Estimation of Vitamin E

2. QUANTITATIVE ANALYSIS OF TOCOPHEROL MIXTURES BY PAPER CHROMATOGRAPHY

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The chemical methods originally used for estimating the vitamin E potency of natural products, notably those of Emmerie & Engel (1938) and of Furter & Meyer (1939), did not distinguish between the different tocopherols. Since α-tocopherol is the only member of the group which possesses marked vitamin E activity, and since the tocopherol mixtures present in natural products differ widely in composition, failure to differentiate between the individual tocopherols is a considerable disadvantage. The fact that γ- and δ-tocopherols couple with diazonium salts, whereas the α and β compounds do not, has, however, been used to achieve a partial analysis of the individual tocopherols (Quaife, 1944; Weisler, Robeson & Baxter, 1947). More recently, Quaife (1948) has described a method

for the quantitative determination of the individual tocopherols in which the nitroso derivatives of the β, γ and δ compounds are separated by chromatography on zinc carbonate-Celite. The concentration of α-tocopherol in the mixture is then determined by difference following summation of the values obtained for the β-, γ- and δ-tocopherols. In a new approach to the problem, Emmerie (1949) showed that α- and γ-tocopherols may be separated from one another by chromatography on alumina or Floridin Earth.

The advantages of using paper chromatography for the precise, qualitative analysis of tocopherol mixtures occurring in natural products have already been described (Brown, 1952). Application of this method to the quantitative estimation of synthetic and naturally occurring mixtures of tocopherols forms the subject of the present paper.

EXPERIMENTAL

Preparation of solutions for chromatography. Since lipids, sterols and carotenoids interfered with the running of the tocopherols on the chromatograms, it was necessary to remove them from extracts of natural products. Lipids were readily removed by saponification (Tošić & Moore, 1945) and the vitamin A and carotenoids were separated from the tocopherols by adsorption on Floridin Earth (Emmerie & Engel, 1939; Glavind, Kjølhede & Prange, 1942). The removal of sterols presented a more difficult problem, and the method finally selected was to crystallize the sterols from methanol solution at $-10°$. After centrifugation and removal of the mother liquor the crystals were redissolved in warm methanol and the solution again cooled to $-10°$. This process was repeated until the supernatant liquors no longer contained tocopherols and the mother liquors were then combined. Although this method is tedious and does not remove the sterols completely, sufficient material is eliminated to avoid interference on the chromatogram.

Standard solutions. These were prepared by dissolving the synthetic ($\pm$)-tocopherols in absolute ethanol. The solutions were stored in amber-coloured bottles at 2° and the reducing activity tested periodically by the Emmerie-Engel method. Solutions containing more than one tocopherol (e.g. α plus β solution for use in examining wheat products) were most conveniently prepared from the stock solutions.

When solutions of tocopherols which had been stored for considerable periods (about 6 months) were chromatographed (see below) faint pink spots were obtained on the starting line on spraying with 2:2′-dipyridyl and $FeCl_3$. In addition, α-tocopherol solutions which had been stored for considerable periods gave a distinct spot with an R_F value of 0·16 in 75 % (v/v) ethanol as well as an authentic α-tocopherol spot. This value corresponds closely with the R_F value of α-tocopherylquinone under the same conditions (Blaxter & Brown, 1952). When these non-tocopherol spots were obtained on chromatographing the standard solutions fresh standards were prepared.

Chromatography. Whatman no. 1 filter paper was coated with Vaseline according to the method previously described (Brown, 1952), and 75 % (v/v) ethanol was used as the mobile phase. The ethanol was distilled from KOH and $KMnO_4$ before use. Sheets of impregnated paper measuring 30 × 12 cm. were used so that four spots, each 3 cm. from its neighbour, could be accommodated on a starting line drawn parallel to the shorter edge of the paper. Two spots of the unknown solution and two spots of a standard solution, containing approximately the same amounts of individual tocopherols as the unknowns, were used for a single determination. The unknown spots were run in positions 1 and 2, while the standard spots occupied positions 3 and 4. The maximum quantity of any individual tocopherol applied to the paper was restricted to 30 μg. since amounts in excess of this gave elongated spots on the developed chromatograms.

After developing the chromatogram for about 16 hr., using the ascending method of Williams & Kirby (1948), the sheet was dried for 15 min. at room temperature and then cut longitudinally into four strips, each containing the tocopherols from one original spot on the starting line. The alternate strips 2 and 4, representing the standard and the unknown, were sprayed with 2:2′-dipyridyl solution (0·25 %, w/v, in ethanol) and then with $FeCl_3$ solution (0·1 %, w/v, in ethanol). This gave a clear definition of the positions occupied by the individual tocopherols. The sprayed strips were then placed alongside the unsprayed strips and the appropriate 'tocopherol zones' of the latter cut out, extracted with 4 ml. hot ethanol, and 1 ml. benzene added in order to clear the cooled solutions. Blank zones, equal in size to the tocopherol zones, were cut from the strips and treated in the same way. Samples of the filtered ethanol-benzene solutions were then tested with 2:2′-dipyridyl and $FeCl_3$ (Emmerie & Engel, 1938) the colour being measured after 2 min. (Baxter *et al.* 1947).

The above method permitted the quantitative separation of the α and δ compounds. Since the β and γ isomers have the same R_F value under these conditions, the following method was used for their estimation. If no reaction was obtained when the spot in the β plus γ position was sprayed with diazo-*o*-dianisidine, the value obtained by the Emmerie-Engel procedure was taken to be β-tocopherol. If spraying with the diazo solution revealed γ to be present, it was estimated in a duplicate chromatogram by the method of Weisler *et al.* (1947). The difference between the value obtained by the Emmerie-Engel procedure for β- plus γ-tocopherols and the value obtained for the γ isomer was taken to represent β. So far, however, β- and γ-tocopherols have not been found to occur together in the limited material examined.

RESULTS

Recovery of tocopherols from the chromatograms. The recovery of tocopherols from the developed chromatograms was not quantitative, but depended on the length of time of development (see Fig. 1). When the chromatograms were run for 16 hr. the recoveries were about 80 %. By reducing the time of development to 6 hr. the recoveries could be increased to about 90 % but the shorter time of development did not allow a clear-cut separation of the γ and δ compounds.

The variation in the recovery with alteration in the time of development suggested that the atmosphere in the chromatogram chamber was a possible cause of destruction of the tocopherols.

Filling the chamber with nitrogen gas after closing the chamber did not, however, decrease the losses. In addition, tocopherols spotted on to the paper above the greatest height reached by the solvent could be recovered quantitatively after 24 hr. exposure to chamber atmosphere. It appeared likely, therefore, that the destruction was caused by a non-volatile constituent of the solvent, but attempts to eliminate the contaminant by distillation were unsuccessful. Use of alternative solvents such as aqueous methanol or acetic acid likewise

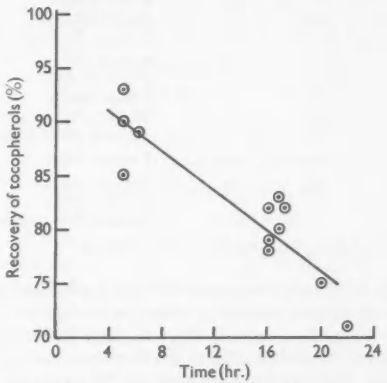

Fig. 1. The effect of time of development on the recovery of tocopherols from the chromatograms.

gave non-quantitative recoveries. In fact, with 90% (v/v) acetic acid prepared from different batches of Analar glacial acetic acid the recoveries ranged from 30 to 80% and were not altered by distillation.

With natural products failure to obtain quantitative recovery of the tocopherols from the developed chromatograms was allowed for by running standards alongside each unknown solution, as described in the Experimental section. As a further check, the recovery of tocopherols in an unknown solution was calculated from the potency of the solution applied to the starting line compared with the sum of the potencies of the individual spots (tocopherols plus artifacts) on the chromatogram. Recoveries of tocopherols from unknown and standard solutions agreed to ± 5% when chromatographed under identical conditions on the same sheet, as for example in *a*, *b* and *c* in Table 1.

Examination of vegetable oils. The tocopherol content of vegetable oils has been examined quite extensively in the past, mainly because they are relatively rich sources of these compounds. It was thought that their examination offered the best means of comparing the present method with those previously published (e.g. Quaife, 1948). In Table 2 the results obtained in the present investigation are compared with the values of other workers. It can be seen that the results are in good agreement, considering that entirely different samples of oil were used by the different workers. In cases where non-tocopherol spots were obtained (e.g. wheat-germ oil) the reducing power of the artifact (calculated as tocopherol) is indicated. The artifact occurring in the wheat-germ oil examined in the present work contributed 9% to the reducing power of the extract applied to the chromatogram. The nature of the artifact was not ascertained, but it was not carotenoid in nature since repeated passage through Floridin Earth columns did not remove it. A similar observation was made with a sample of shark-liver oil; in this case the artifact contributed 43% of the total reducing power of the extract applied to the chromatogram. The potential error which is present in the estimation of the vitamin E potency of natural products is thus clearly demonstrated.

Table 1. *Recovery of tocopherols from paper chromatograms*

Test	Solution	Reducing power applied (calc. as μg. tocopherol)	Recovery (%)
(*a*)	α-Tocopherol	18·8	78
	β-Tocopherol	10·8	79
	Wheat-germ oil*	36·0	77
(*b*)	α-Tocopherol	18·8	84
	γ-Tocopherol	11·8	75
	Cottonseed oil†	24·4	78
(*c*)	α-Tocopherol	17·2	81
	γ-Tocopherol	21·6	77
	δ-Tocopherol	12·8	85
	Soya-bean oil‡	59·2	81

* The wheat-germ oil contained a mixture of α- and β-tocopherols and artifact.
† The cottonseed oil contained a mixture of α- and γ-tocopherols.
‡ The soya-bean oil contained a mixture of α-, γ- and δ-tocopherols.

Table 2. *Tocopherol content of vegetable oils*

Oil	Total tocopherol (mg./100 g.)	Individual tocopherols (% of total) α-	β-	γ-	δ-	Artifact	
Corn (Maize)	86	6	0	93	0	1	Present work
	90	10	—	90	—	—	Fisher (1945)
	102	12	0	88	0	—	Quaife (1948)
	104*	6	—	—	—	—	Hove & Hove (1944)
Cottonseed	70	74	0	26	0	0	Present work
	87	69	—	31	—	—	Fisher (1945)
	95	75	—	25	—	—	Fisher (1945)
	110	69	—	31	—	—	Fisher (1945)
	87	57	0	43	0	—	Quaife (1948)
	83*	62	—	—	—	—	Hove & Hove (1944)
Soya bean	83	15	0	51	34	0	Present work
	94	21	—	79	—	—	Fisher (1945)
	99	21	—	79	—	—	Fisher (1945)
	74	13	0	56	31	—	Quaife (1948)
	92*	10	—	—	—	—	Hove & Hove (1944)
Sunflower seed	51	100	0	0	0	0	Present work
Wheat germ	262	64	27	0	0	9	Present work
	268	60	40	—	—	—	Quaife (1948)
	274*	70	—	—	—	—	Hove & Hove (1944)

* In the work of Hove & Hove (1944) only the total and the α-tocopherol were determined.

DISCUSSION

Since the original chemical methods for determining vitamin E in natural products (Emmerie & Engel, 1938; Furter & Meyer, 1939) do not distinguish between the individual tocopherols, which differ markedly in biological activity, comparison between chemical and biological methods is virtually impossible. Almost all the methods for the determination of the individual tocopherols in natural products (Quaife, 1944, 1948; Weisler *et al.* 1947) have the disadvantage that the concentration of α-tocopherol, the most biologically active member of the vitamin E group, is estimated by difference.

The present method allows each of the individual tocopherols to be determined separately and simultaneously, and has the additional advantage that it is applicable to microgram quantities of tocopherols. This is an important consideration in the examination of animal tissues, which frequently contain only small amounts of tocopherols. In addition, the presence of non-tocopherol reducing substances in the extracts of natural products is readily demonstrated by this method. On the other hand, small losses of the tocopherols occur in paper chromatography, but this disadvantage can be overcome by running standards alongside each unknown solution.

SUMMARY

A method involving paper chromatography has been developed for the quantitative estimation of the individual tocopherols (α-, β-, γ- and δ-). Results obtained by this method for certain vegetable oils have been compared with those obtained for the same oils by other workers.

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The Conversion of Citrate into *cis*-Aconitate and *iso*Citrate in the Presence of Aconitase

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Martius & Lynen (1950) and Friedrich-Freksa & Martius (1951) have expressed doubts on the intermediary formation of *cis*-aconitate in the aconitase system because a study of the kinetics of the conversion of citrate into *iso*citrate failed to show a lag period. The authors argue that if the conversion followed the original scheme of Martius (1937)

citrate ⇌ *cis*-aconitate ⇌ *iso*citrate

the rate of formation of *iso*citrate should gradually rise until the optimal concentration range of *cis*-aconitate was built up, and they conclude from the absence of a lag period in their experiments that the interconversion of citrate and *iso*citrate does not necessitate the formation of *cis*-aconitate.

The experiments reported in this paper were designed to re-investigate the question of the existence of a lag period in the aconitate system. Dilute heart-muscle aconitase preparations were mixed with citrate, and the time course of the formation of aconitate and *iso*citrate was measured at 25°. Dilution and a relatively low temperature draws out the time curve and thus favours the detection of a lag period.

EXPERIMENTAL

Pig heart (17 g.), minced in a domestic mincer, was suspended for 5 min. in 20 vol. 0·9% NaCl. The supernatant was removed by centrifugation and the washed muscle was homogenized with 250 ml. water in a Waring Blendor. The object of washing the muscle was to remove substances, mainly phosphates, lactic acid and unsaturated compounds, which interfere with the determinations of either *cis*-aconitate or *iso*citrate. A mixture of 120 ml. heart-muscle suspension, 120 ml. M-sodium citrate, 150 ml. 0·1 M-$NaHCO_3$ and 210 ml. water was incubated at 25° with continuous mechanical stirring. A stream of 5% CO_2 in N_2 (v/v) was passed through the suspension throughout so that pH was 7·4. A control suspension contained the same reagents except citrate. At intervals 25 ml. samples were removed for analysis. The sample for the determination of aconitate was acidified with 6·5 ml. 20% (w/v) metaphosphoric acid, that for the determination of *iso*citric acid with 2·5 ml. glacial acetic acid. These quantities were sufficient for duplicate analyses.

Aconitic acid was determined manometrically according to Johnson (1939) by quantitative catalytic hydrogenation. The acid was extracted with ether from a 13 ml. sample in a Kutscher-Steudel apparatus after addition of 2 ml. 50% (w/v) H_2SO_4. The extracted material, after evaporation of the ether, was dissolved in a total volume of 2·5 ml. water. The main compartment of a conical Warburg manometer contained 25 mg. of the palladium catalyst of Köppen (1932) and 3 ml. 3% (w/v) metaphosphoric acid, the side arm 0·9 ml. of the ethereal extract and 0·1 ml. 20% (w/v) metaphosphoric acid. The gas space contained cylinder hydrogen washed with pyrogallol and acid dichromate. The bath temperature was 38°.

*iso*Citric acid was determined polarimetrically. To 27·5 ml. of the acidified solution 22·5 ml. 29% (w/v) ammonium molybdate were added. After filtration the rotation was measured at 18° in 2 dm. tubes. The concentration of *iso*citrate was calculated on the basis of the $[\alpha]_D$ values given by Eggleston & Krebs (1949).

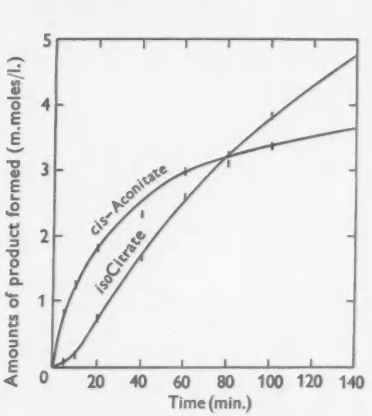

Fig. 1. Time curve of the formation of *cis*-aconitate and *iso*citrate from citrate in the presence of aconitase. For conditions see text. Additional data: amounts of *cis*-aconitate after 180 min. 4·16, after 270 min. 4·25, after 24 hr. 5·80 m-moles/l.; amounts of *iso*citrate after 180 min. 6·02, after 270 min. 8·08, after 24 hr. 12·4 m-moles/l.

The results of a representative experiment are as in Fig. 1. The curves clearly demonstrate the existence of a lag period before the formation of *iso*-citrate reaches maximum velocity. Hardly any

*iso*citrate is formed in the first 5 min. The rate rises progressively and reaches a maximum after about 40 min. In contrast, the velocity of *cis*-aconitate formation is maximal at the start and decreases gradually. Similar curves were obtained in five other experiments. They are in accordance with the assumption that *cis*-aconitate is an intermediate in the conversion of citrate into *iso*citrate and there is therefore no reason for changing the theory of aconitase action originally put forward by Martius (1937).

SUMMARY

The rate of formation of *iso*citrate and *cis*-aconitase from citrate in the presence of heart-muscle aconitase was measured. Contrary to findings by Friedrich-Freksa & Martius (1951) the rate of *iso*-citrate formation was not maximal at the start, but showed a distinct lag period. The observations are in agreement with Martius's earlier assumption (1937) that *cis*-aconitate is an intermediate in the conversion of citrate into *iso*citrate.

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